An Examination of the Possible Role(s) of Disulphide Exchange in the Regulation of Integrin Function

By

Yanglong Mou

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For the Degree of

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FACULTY OF GRADUATE STUDIES

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An examination of the possible role(s) of disulphide exchange in the regulation of integrin function

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LIST OF PUBLICATIONS

1. Ni HY, Mou YL, McGlaughlin-Puzon W, Takada Y and Wilkins JA: "Evidence for long range and intracellular regulation of the exposure of a cation and ligand induced binding site (CLIBS) on beta-1 integrin". Submitted to Journal of Immunology.

2. Mou YL, Ni, HY and Wilkins JA: Selective inhibition of β1 and β7 integrin by Bacitracin. J. Immunol. 1998, 161: 6323.

3. El-Gabalawy HS, King R, Bernstein C, Ma GP, Mou YL, Alguacil-Garcia A, Fritzler M and Wilkins JA: Expression of N-Acetyl-D-Galactosamine associated epitope in synovium: a potential marker of glycoprotein production. J. Rheumatol. 1997, 24: 1355.

4. Bernsten CN, Pettigrew NM, El-Gabalawy HS, Sargent M, Mou YL and Wilkins JA: The differential expression of a novel intestinal epithelial glycoprotein in various forms of inflammatory bowel disease. Am. J. Clin. Pathol. 1996, 106: 42.

LIST OF ABBREVIATIONS USED

APCs	Antigen presenting cells
ATCC	American type culture collection
BCN	Bacitracin
BSA	Bovine Serum Albumin
CLIBS	Cation and Ligand-influenced Binding Site
COL	Collagen
CS1	Connecting Segment 1 (alternatively spliced) of FN
DMSO	Dimethyl Sulfoxide
DTNB	Dithiobisnitrobenzoic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetracetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FAK	Focal adhesion kinase
FACS	Fluorescence-activated cell-sorting
FAT	Focal adhesion targeting
FBS	Fetal bovine serum
FN	Fibronectin
FN-40	The 40 KD fragment of FN containing LDV
FN-120	The 120 KD fragment of FN containing RGD
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
HPLC	High pressure liquid chromatography
ICAM	Intercellular adhesion molecule
ICAP	Integrin cytoplasmic domain-associated protein
IGSF	Immunoglobulin superfamily
IL	Interleukin
ILK	Integrin-linked kinase
LABS	Ligand-attenuated binding site

LDV	Leucine-Aspartate-Valine peptide
LFA	Lymphocyte function associated antigen
LN	Laminin
mAb	Monoclonal antibody
MAdCAM	Mucosal addresin cell adhesion molecule
MMP	Matrix metalloproteinase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PHA	Phytohemagglutinin
PMA	Phorbol myristyl acetate
PMSF	Phenylmethylsulfonyl fluoride
RGD	Arginine-Glycine-Aspartate peptide
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
VCAM	Vascular cell adhesion molecule

Abstract

Integrins are heterodimeric type 1 transmembrane proteins which are involved in cell-cell and cell-matrix interactions. At present the regulation of the functional status of integrins on the cell surface is unclear. During the investigation of the possible role of protein disulfide isomerase (PDI) in the regulation of integrin functions, bacitracin was found to be capable of inhibiting β1 integrin functions. It was demonstrated that (1) Bacitracin could inhibit α2β1 integrin-mediated cell adhesion to collagen, α4β1 and α5β1 integrin-mediated cell adhesion to fibronectin, α6β1 integrin-mediated cell adhesion to laminin. (2) Bacitracin did not cause changes in the expression level of integrin on the cell surface. (3) It upregulated the expression of Cation and Ligand-Influenced Binding Site(s) (CLIBS) on β1 integrins, indicating that bacitracin treatment could cause changes in integrin conformation. (4) Bacitracin could inhibit soluble fibronectin binding to living cells and to purified integrin. (5) Bacitracin induced expression of CLIBS is a divalent cation independent which is different from that caused by integrin ligands. (6) Double reciprocal data showed that bacitracin may cause allosteric inhibition of integrin functions.

Based on these results a model of bacitracin induced inhibition of integrin function is proposed in which bacitracin may interact directly with integrin and cause allosteric changes to integrin, thus inhibiting integrin function. Data are also presented showing that bacitracin can selectively inhibit β2, β7 but less extent to β3 integrin-mediated cell adhesion. These observations may be important for the further exploration

of the regulatory mechanisms of integrin function. Bacitracin may serve as a useful probe for research in this area.

Introduction

1. Adhesion control is critical for immune functions.

Lymphocytes are very important cells in host defense against invading microorganisms. Lymphocytes constantly recirculate between lymphoid organs and other tissues via lymph and blood. This process plays a key role in their immune surveillance function (Weissman IL and Cooper MD, 1993; Paul WE, 1993).

In healthy individuals, lymphocytes continuously recirculate from blood to lymphoid organs and back to the blood via the lymphatics. Binding avidity of circulating lymphocytes to low vascular endothelium is very weak (Kraal G and Mebius RE, 1997). This characteristic not only correlates with their surveillance function of the immune system but also allows them to quickly migrate to the regions of microbial deposition. In lymphatic tissues, lymphocytes migrate through lymphatic tissues in search of their cognate antigens presented as antigen-MHC complexes on antigen-presenting cell surfaces in the tissues. Low avidity of lymphocytes to the vascular endothelium allows lymphocytes to be able to move throughout the body, and accumulate rapidly in the sites of microbial attack (Salmi M and Jalkanen S, 1997). However, the behavior of lymphocytes is completely changed once a pathogen has entered the body and an inflammatory/immune response is initiated. Lymphocytes immediately undergo a change from a motile state to a highly adhesive one. These cells must first adhere to the vascular endothelium adjacent to the regions of infection, pass through the endothelium, and migrate directly toward the sites of pathogen deposition (Dunon D et al, 1993).

Adhesion control is also important for immune activation. Immune responses require interactions among lymphocytes and between lymphocyte and antigen-presenting cells (APCs). Normally, both lymphocytes and APCs in tissues are in a "resting" state and have low avidity for each other. In this condition, if such APCs present self-antigens to specific T cells, the T cells may become anergic. This characteristic maintains immune cells as self-tolerant and prevents autoimmune reactions against the tissue antigens (Kruisbeek AM and Amsen D, 1996). However, microbial infection may activate resident APCs, leading to increased expression of costimulators and cytokines that stimulate T cells (Germain RH and Marguiles DM, 1993). Activated T cells show high avidity to their cognate APCs presenting antigen-MHC complex on their surface, and the interaction between T cells and APCs allows specific immune response to occur, which finally lead to elimination of the microbe (Germain RH and Marguiles DM, 1993).

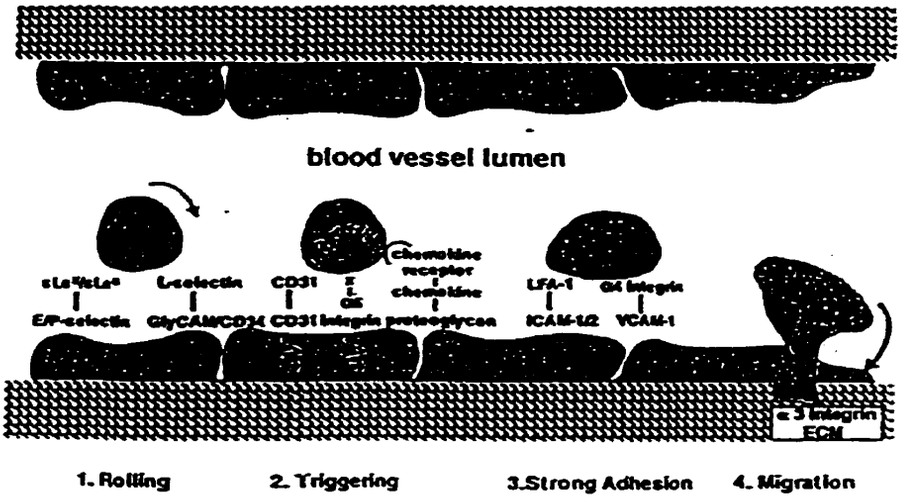
2. Adhesion molecules are strictly controlled in the adhesion cascade.

During inflammation, the activated lymphocytes tend to migrate to the sites of inflammation during their recirculation. The activated lymphocytes must first interact with endothelial cells lining in the blood vessels and transmigrate into the surrounding tissue. These processes are called lymphocyte extravasation. The transmigrated lymphocytes then migrate directly to the site of inflammation (Imhof BA et al, 1996). The lymphocyte transmigration process can be generally divided into four steps (Kraal G et al., 1997; Salmi M et al., 1997; Imhof BA et al., 1996) (Figure 1-1): (1) *Rolling and tethering* The interactions between selectins and their ligands are very important for rolling and tethering. The interaction between $\alpha 4\beta 1$ integrin with VCAM-1, $\alpha 4\beta 7$

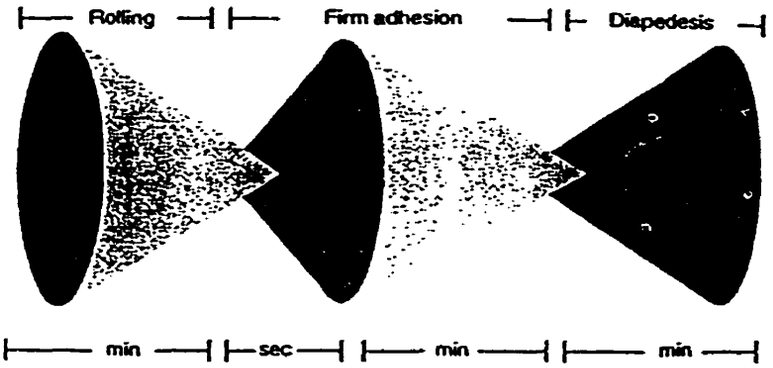
integrin with MadCAM-1 and possibly α9β1 integrin with VCAM-1 (Carlos TM and Harlan JM, 1994; Taooka Y, 1999) are also involved. (2) *The triggering of adhesion molecules on the cell surface by the interaction between chemokines and chemokine receptors* This is a very rapid process (Campbell JJ et al, 1998). (3) *The establishment of firm adhesion to the endothelium by the interaction between integrin α4β1, α4β7 and αLβ2 with their ligands (Ley K and Tedder TF, 1995)* It is this strong adhesion that can rapidly bring flowing T cells to a halt. (4) *Transmigration into surrounding tissues* CD31 is involved in lymphocyte transmigration (Muller WA et al, 1993). The triggering signals from CD31 and chemokines to integrins may be decreased rapidly. At the same time selectins are shed from the cell surface. This allows lymphocytes to transmigrate into tissues by transitioning from one adhesive system to another (Salmi M et al, 1997; Ley K and Tedder TF, 1995).

The lymphocyte recirculation patterns in naive and effector lymphocytes are different. This difference is related to the expression of adhesion molecules and their functional states on both lymphocytes and endothelial cells (Salmi M and Jalkanen S, 1997; Springer TA, 1994). Naive lymphocytes are randomly distributed in different lymphatic tissues. They can reenter the same or a different lymphatic tissue. During inflammation, when a lymphocyte encounters its antigen it halts in the secondary lymphatic organ and undergoes clonal expansion and differentiation. The recirculation of the progeny of the effector cells is restricted to the sites of original antigenic insult and related lymphoid tissues (Salmi M and Jalkanen S, 1997).

A.



B.



	selectins	chemokine activation	integrin activation	integrin activation chemoattractants
Peripheral lymph node HEV	L selectin - GlyCAM-1 L selectin - CD34 L selectin - sgp200	G protein linked receptors	LFA-1 - ICAM-1	CD31 chemoattractants ?
Peyer's patch HEV	α4β7 - MAdCAM-1 L selectin - MAdCAM-1	G protein linked receptors	LFA-1 - ICAM-1 α4β7 - MAdCAM-1	CD31 chemoattractants ?
Venules at inflammation site	α4β1 - VCAM-1 CD44 - hyaluronate E selectin - ESL-1 P selectin - PSGL-1 L selectin - sLex? VAP-1 - ?	G protein linked receptors	LFA-1 - ICAM-1 α4β1 - VCAM-1	CD31 chemoattractants ?

Figure 1-1: Four steps of lymphocyte transmigration process. (A)The four steps are rolling or tethering, triggering, strong adhesion, and migration. (B)The width of the conus indicates the importance of a given process and its reversibility over time. The major molecular interactions involved in the various processes in peripheral lymph nodes, Peyer's patches, and at sites of inflammation are listed (From Imhof BA et al., 1995; Kraal G et al., 1997).

In summary, both lymphocytes and endothelial cells must stringently control adhesion molecules on their surface in order to regulate the adhesion cascade.

Although it is apparent from the previous discussions that multiple adhesion molecules are required for effective lymphocyte recruitment, activation and function, this research specifically focuses on members of the integrin family. These molecules play central roles in each of these processes. The integrins mediate adhesion to cellular and extracellular ligands. As such they represent a useful system for the analyses of adhesive mechanisms and their regulation. These comments are meant to highlight the importance of the integrins rather than to suggest that other adhesion molecules (e.g. Selectins, Cadherins, Immunoglobin superfamily and Sialomucins) are not key elements in immune function.

3. Integrins are involved in many biological processes

3.1 Integrin structure

The integrins are a family of type I transmembrane glycoproteins which possess a variety of common structural and functional characteristics. They consist of two non-covalently linked chains, the α and β-subunits, and have been subclassified on the basis of their β-unit (Hillis GS and Macleod AM, 1996).

Sixteen α subunits and eight β subunits of integrin have been identified so far. Each α and β subunit may potentially form different combinations to generate 22 different pairs (Figure 1-2). They have different ligand specificities and are involved in different biological processes (Table 1-1) (Boudreau NJ and Jones PL, 1999).

Integrin receptors exhibit considerable overlap in their ligand-binding specificity. For example, α3β1 can bind to fibronectin, laminin and collagens, while fibronectin is the ligand for α4β1, α5β1, αvβ3 and αvβ6 integrins (Hynes RO, 1992). This multiplicity of receptors indicates that evolution has provided a complex compensatory system. For example, cells lacking α5β1 integrin can use other β1 (Yang JT et al 1993) or β3 (Wennenberg K et al 1996) integrins to bind fibronectin. On the other hand, distinct receptors that bind to the same ligand are able to trigger different cellular responses in several cases. The α6β1 and α6β4 integrins both can bind to laminin. However, they are obviously different in their interactions with cytoskeletal proteins, and in the ability to activate the MAPK pathway via Shc (Wary KK et al, 1996). Thus, cells may use different receptors for a given matrix molecule to allow differentiation of the cellular response.

Because of different splice variants of several subunits, some integrin subunits may have different isoforms. β1 integrins have been found to have at least four isoforms, β1A, β1B, β1C and β1D (Altruda F et al, 1990; Belkin AM et al, 1996; Languino LR and Ruoslahti E, 1992; Zhidkova NI et al 1995). These all have the same extracellular domain and transmembrane regions but unique COOH terminal regions. These differences in cytoplasmic regions may result in different physiological functions. β1A is the ubiquitous integrin β subunit. When compared with β1A, β1C has been reported to inhibit cell cycle progression (Meredith J et al, 1995), while β1B does not activate signaling through P125 FAK nor does it organize focal adhesion (Balzac F et al, 1993), β1D shows a high capacity to bind matrix ligands and cytoskeletal components of the focal adhesions such

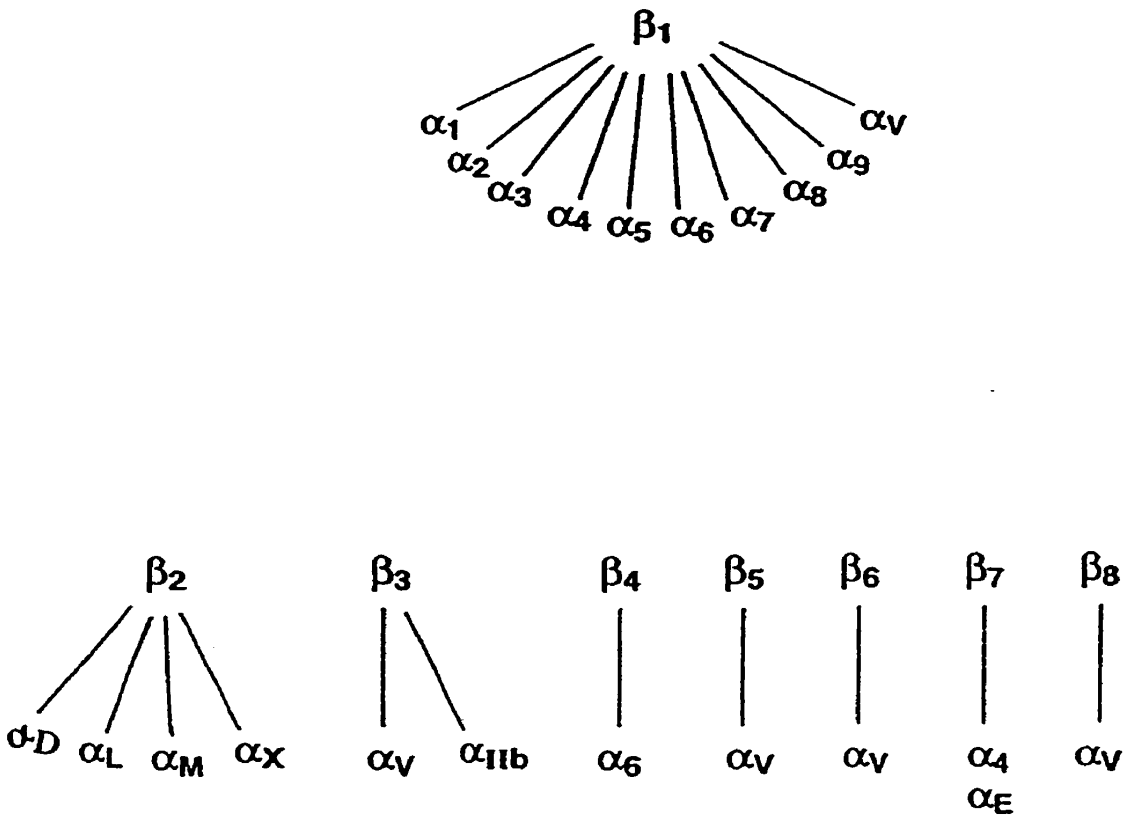

β1
α1
α2
α3
α4
α5
α6
α7
α8
α9
αV
β2
αD
αL
αM
αX
β3
αV
αIIb
β4
α6
β5
αV
β6
αV
β7
α4
αE
β8
αV

Figure 1-2. Subunit associations within the integrin family (From Kumar CC, 1998). The known combinations of α and β subunits that form integrins are shown.

Table 1-1: The integrin family and its ligands (From Horton MA, 1996)

Receptor		Ligand		Distribution
$\beta 1$	VLA-1	$\alpha 1/\beta 1$	Laminin, collagen	Broad
	VLA-2	$\alpha 2/\beta 1$	Laminin, collagen	Broad
	VLA-3	$\alpha 3A/\beta 1$	Laminin, collagen, fibronectin, epiligrin	Broad
		$\alpha 3B/\beta 1$	Laminin, collagen, fibronectin, epiligrin	Broad
	VLA-4	$\alpha 4/\beta 1$	Fibronectin (V25), VCAM-1	B and T lymphocytes, macrophages, neural crest cells
	VLA-5	$\alpha 5/\beta 1$	Fibronectin (RGD)	Broad
	VLA-6	$\alpha 6A/\beta 1$	Laminin	Broad
		$\alpha 6B/\beta 1$	Laminin	Broad
	VLA-7	$\alpha 7A/\beta 1$	?	?
		$\alpha 7B/\beta 1$	Laminin	?
	VLA-8	$\alpha 8/\beta 1$	?	?
		$\alpha V/\beta 1$	Fibronectin, vitronectin	Epithelial cells
$\beta 2$	LFA-1	$\alpha L/\beta 2$	ICAM-1, ICAM-2, ICAM-3	Leukocytes
	CR3, Mac-1	$\alpha M/\beta 2$	C3bi, factor X, fibrinogen, ICAM-1	Granulocytes, macrophages, natural killer cells, cytotoxic T lymphocytes
	p150,95	$\alpha X/\beta 2$	C3bi, fibrinogen	Macrophages, granulocytes, actived B lymphocytes
$\beta 3$	gpIIb/IIIa	$\alpha IIb/\beta 3$	Fibrinogen, fibronectin, vW factor, vitronectin, thrombospondin	Platelets
	VNR	$\alpha V/\beta 3$	Fibrinogen, fibronectin, vW factor, vitronectin, thrombospondin, osteopontin	Endothelial and tumor cells Osteoclasts
$\beta 4$		$\alpha 6A/\beta 4$	? (Laminin)	Epithelial cells
		$\alpha 6B/\beta 4$	? (Laminin)	Epithelial cells
$\beta 5$		$\alpha V/\beta 5$	Vitronectin	Carcinoma cells
$\beta 6$		$\alpha V/\beta 6$	Fibronectin	?
$\beta 7$		$\alpha 4/\beta 7$	Fibronectin, VCAM-1, MadCAM-1	Activated B and T lymphocytes, macrophages, intraepithelial lymphocytes
		$\alpha E/\beta 7$	?	
$\beta 8$		$\alpha B/\beta 8$	?	?
		$\alpha 9/\beta 8$	?	?

as talin (Belkin AM et al, 1996). Alternative splicing also occurs in α subunits. Isoforms of α3 (Tamura RN et al, 1991), α6 (Cooper DT et al, 1991), and α7 (Collo G et al, 1993) have been identified.

The structures of different α or β subunits are not identical, but each set of chains has a number of common characteristics (Hillis GS and Macleod AM, 1996).

The α subunits contain about 900-1100 amino acids and most of the sequences are exposed at the extracellular space. The cytoplasmic domain consists of 15-50 amino acid residues. The extracellular domain of the α subunit is composed of seven repeated regions. The last three or four repeated regions are thought to contain EF hand-type-like domains that bind the divalent cations Ca^{++} or Mg^{++} (Kawasaki H and Kretsinger RH, 1995). The α subunits (for example GPIIb) contain 18 Cysteines which form 9 disulfide bonds (Wang R et al, 1997). There is little sequence homology among α subunits except for a GFFKR motif, which is located near the transmembrane region of the intracellular domain (Schwartz MA et al, 1995). The cytoplasmic domain of α subunit is involved in the signal transduction through integrin. The GFFKR motif is thought to be the binding site of calreticulin. Removing the GFFKR sequence causes constitutive activation of integrin (Crow DT et al 1994; Hughes PE et al, 1995), suggesting the important roles of this region in the regulation of integrin function.

Some α subunits are post-translationally cleaved to give a 25-30 kD transmembrane fragment that is disulfide-bonded to a larger, wholly extracellular chain. Other α subunits contain an extra segment of approximately 180 amino acids called I (inserted) domain which is located between repeated regions II and III. I domains are characteristic of α1, α2, αL, αM, αX, αD and αE (Takada Y et al, 1997). Growing

evidence indicates that the I domain participates in ligand binding. Function-altering antibodies, both stimulatory and inhibitory, map to I domains of αMβ2 (Diamond MS et al 1993, Michishita M et al 1993), αLβ2 (Landis R et al, 1993; Randi AM and Hogg N, 1994), α2β1 (Kamata T et al, 1994) and α1β1 (Kern A et al, 1994). Mutations in I domain block a ligand-binding function (Kamata T et al 1994; Kern A et al 1994). The recombinant I domain from αM, α2 and αL fusion proteins binds to ICAM-1 or collagen (Kamata T and Takada Y, 1994; Randi AM and Hogg N, 1994; Zhou L et al, 1994). The conserved Asp residues in the I-domain (Asp154, Thr221 and Asp252 in α2) have been identified as critical for ligand binding (Kamata T et al, 1995). The I domain of αM also binds cations, which appear to be important in I domain ligand-binding function (Michishita M et al 1993). In I domain crystal structures, the divalent cations are directly coordinated at the cation binding site by residues Ser139, Ser141 and Asp239 of αL. (Qu A and Leahy DJ, 1995; Qu A and Leahy DJ, 1996).

Figure 1-3 shows schematic stereoribbon diagram of integrin α subunit structure (Chothia C and Jones EY, 1997). The I domain is composed of alternating amphipathic α helices and hydrophobic β strands. The five parallel and one antiparallel β strands form a central sheet that is surrounded by the seven α helices. A crevice which is the ligand binding site in I domain is formed along the top of the folded structure (Dickeson SK and Santoro SA, 1998).

α subunits have ligand binding N-terminal domains. The N-terminal domain in α subunit has a seven-fold repeat, with each repeat consisting of about 60 residues and is predicted to form a four-stranded β-sheet, and the seven β-sheets from the seven repeats

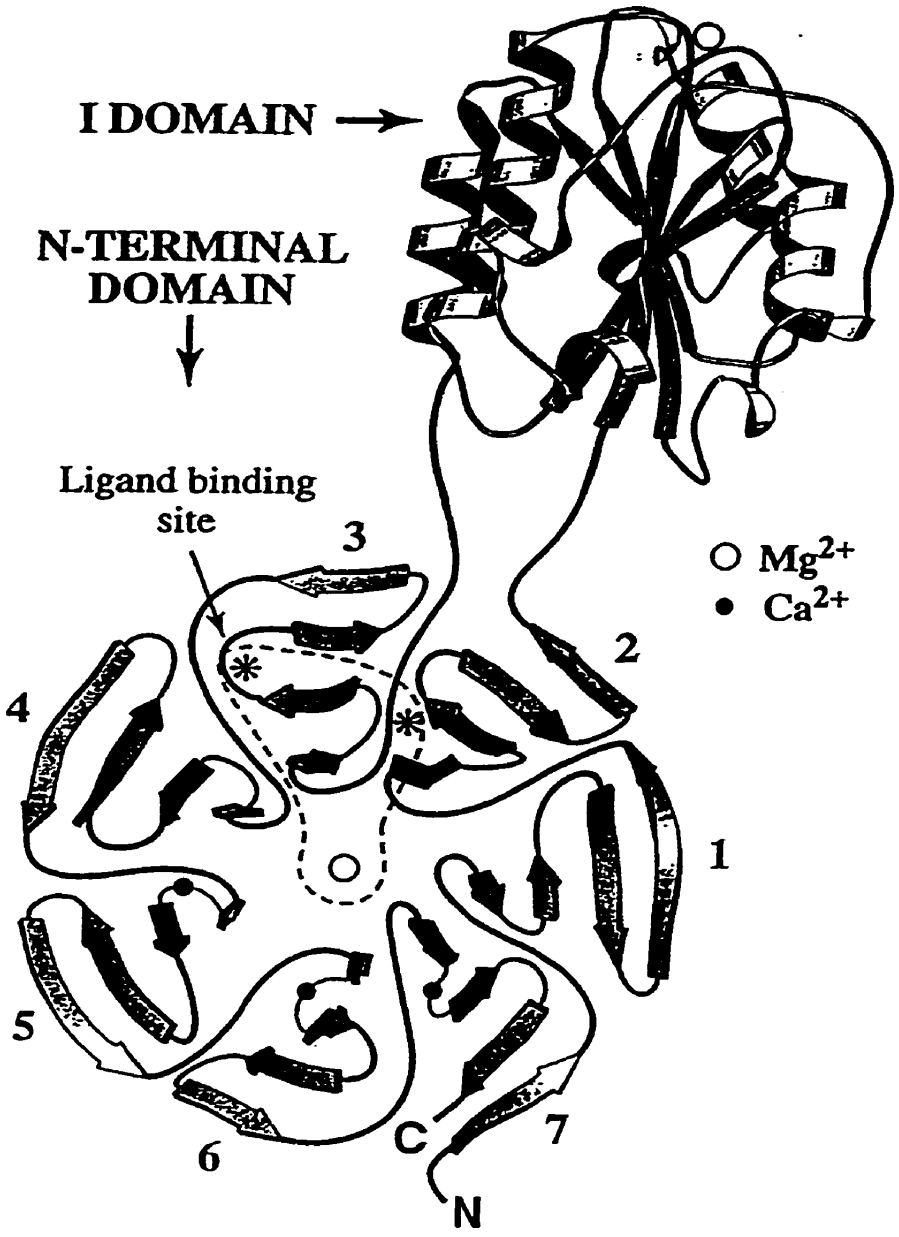

I DOMAIN
N-TERMINAL DOMAIN
Ligand binding site
3
2
4
1
5
6
C
7
N
O Mg2+
• Ca2+

Figure 1-3: α subunit of integrin (Chothia C and Jones EY, 1997). Top: the observed structure of the I domain. Bottom: a schematic drawing of the β-propeller structure predicted for the ligand-binding N-terminal domain. Thick ribbons represent strands of β-sheet, and coiled ribbons represent α-helices.

are predicted to have a pseudosymmetric radial arrangement that gives a β-propeller fold (Chothia C and Jones EY, 1997).

β subunits contain between 650-800 amino acids except β4, and a large portion of the molecule is exposed at the extracellular surface with only 40-50 residues located in the cytoplasmic domain. The β4 subunit consists of 1752 residues, 1000 of which contribute to the cytoplasmic domain (Suzuki S and Naitoh Y, 1990). The β subunits contain 56 extracellular cysteine residues whose positions are highly conserved among different β subunits as well as between different species such as Drosophila and human molecules (Hemler ME, 1990). Among the different β subunits, the disulfide bonds paired by intrachain cysteine residues of GPIIIa are the most fully characterized. All of the cysteine residues in GPIIIa are normally disulfide-linked to form 28 nonconsecutive disulfide bridges. Two large loops extending from amino acids Cys5-Cys435 and Cys406-Cys655 have been proposed in GPIIIa subunit (Calvete JJ et al, 1991). A cysteine-rich region in the extracellular domain of the β subunit is folded to form four cysteine-rich motifs. However, recent paper by Yan B and Smith JW indicates that free cysteine residues located in cysteine-rich region exist in both activated and resting GPIIIa subunit (Yan B and Smith JW, 2000). The functions of these conserved cysteines and their disulfide bonds are unclear. One possibility is that this high content of disulfide bonds may enable integrins to change their conformation by altering intrachain or interchain disulfide bonds or even between integrins and ligands. This may provide a basis for regulating the functional states of integrins and cell adhesive-nonadhesive states.

Based on the similarity of hydropathy profiles between the I-domain and part of the β subunit, an I-domain-like structure within the β subunit has been proposed (Figure

1-4) (Tuckwell D and Humphries M, 1997). In this model, the upper face of the I-domain-like structure of the β subunit is predicted to be one of the ligand binding sites containing a diverse disulfide-linked sequence in different β chains surrounded by conserved oxygenated residues critical for ligand binding (Asp130 in β1 integrin for example). The antibody regulatory site (residues 207-218), which is reported to be recognized by both stimulatory and inhibitory anti-β1 integrin mAbs (Takada Y and Puzon W, 1993) is located in the small region in a predicted loop on the opposite side of the domain (Tuckwell D and Humphries M, 1997).

The α and β subunits dimerize to form a heterodimer that, by electron microscopy, appears as a globular head with two extended stalks (Hynes RO, 1992). The extracellular domains of the two subunits likely contribute to the globular head region while the two stalks extend to the lipid bilayer. It appears that the association of the two subunits is regulated at least in part by interaction of the extracellular domains as truncated forms of both α and β subunits with only extracellular domains can form heterodimers (Dana N et al, 1991).

The sites on the integrin α subunit involved in ligand binding, in general, are not well characterized. The extracellular domain of the α subunit is composed of seven homologous, repeated domains. The repeats III on α4 and repeats V-VII (or IV-VII in some integrins) that resemble the classical Ca^{++} binding motifs known as EF-hands on α4 integrin may be involved in the ligand binding since point mutations in these regions impair ligand binding (Irie A et al, 1995; Masumoto A and Hemler ME, 1993). Cao Z et al identified a 144-residue region (positions 223-367) on the α5 subunit as a putative binding region by chimeric receptors (Cao Z et al, 1998). Residues 294-314 of αIIb that

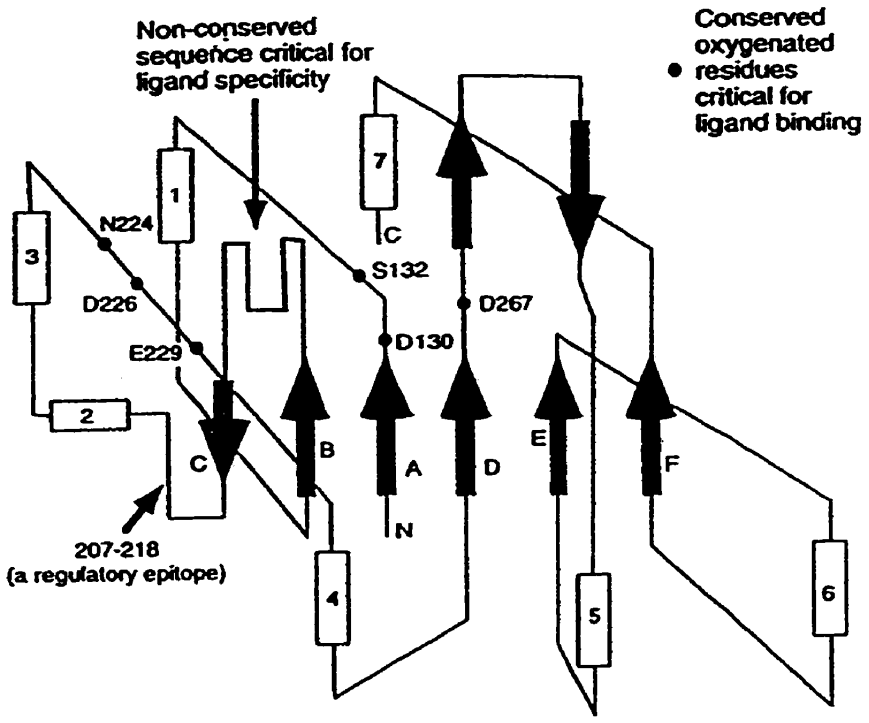

Non-conserved
sequence critical for
ligand specificity
Conserved
oxygenated
residues
critical for
ligand binding
1
2
3
4
5
6
7
N224
D226
E229
S132
D130
D267
C
N
A
B
C
D
E
F
207-218
(a regulatory epitope)

Figure 1-4: Hypothetical model of the I-domain-like structure of the β subunit (Takada Y et al, 1997). Arrows indicate β-sheets and columns indicate α-helices.

contains a putative divalent cation-binding site appears to be critical for the ligand recognition (D'Souza SF et al 1990). Peptides from this region (296-306) can inhibit fibrinogen binding to αIIbβ3 integrin, as do antibodies against the peptides (D'Souza SF et al 1991). Mutational analyses of these regions in αIIb subunit have been hampered by interference with integrin biosynthesis (Wilcox DA et al 1994). The domain of the integrin β subunit involved in determining ligand binding specificity has been identified. Lin EC et al expressed chimeras of β3 and β5, the most homologous integrin β subunits, with αv subunit on human 293 cell surface. The ligand binding phenotype of each chimera was assessed using the ligands Fab-9 and fibrinogen as both have a binding preference for αvβ3 integrin. They found that a swap of residues 164-202 in β3 integrin into the backbone of β5 integrin enabled the chimeric integrin to bind the β3 ligands. This suggested that this region might play a role in determining ligand selection by all integrins as this 39-residue domain is highly divergent among the integrin β subunits (Lin EC et al, 1997).

The cytoplasmic domains of β subunits interact indirectly with cytoskeletal actin filaments by the protein talin or α-actinin and thereby initiate the assembly of a complex of intracellular attachment proteins that link the integrin to actin filaments in the cell cortex (Hynes RO, 1992). Thus, the cytoplasmic domains of integrins play an important role in the connection of the cytoskeleton with the plasma membrane. Integrins must interact with the cytoskeleton in order to bind cells to the matrix since integrins with mutant cytoplasmic domain of the β subunit still bind to their ligands but no longer mediate robust cell adhesion or cluster at focal contacts. These cytoskeletal attachments

may also help to cluster integrins to produce a stronger aggregate bond (Languino LR and Ruoslahti E, 1992).

3.2 Role of integrins

Integrins are involved in many biological processes such as embryonic development, maintenance of tissue integrity and leukocyte homing. Deficient or reduced expression of integrins has been identified to be associated with several diseases (Table 1-2). For example, a simple defect in the synthesis of one of the molecules involved in the adhesive interaction between leukocytes and the endothelium, the β2 integrin chain, results in leukocyte adhesion deficiency (LAD) syndrome characterized by recurrent bacterial infections from early life. The lack of cell surface heterodimeric integrin expression results in the inability of neutrophils to properly adhere to the vessel wall and migrate to sites of, for example, bacterial infection, which often leads to a series of virulent bacterial infections. This can lead to the early death of these individuals (Harlan J, 1993). Another example of a disease related to integrin malfunction is Glanzmann's Syndrome characterized by an abnormality of platelet morphology and function. The platelets have reduced adhesion and aggregation, leading to abnormal clot retraction following induction of the normal haemostatic process, and hence a tendency to bleed. This has now been shown to be due to mutations in the platelet GPIIbIIIa glycoprotein complex, the integrin that mediates platelet interaction with fibrinogen (Calveta JJ, 1994). Furthermore, while mice heterozygous for β1 integrin ($\beta1^{+/-}$) appeared to be normal, homozygous inactivation of the gene ($\beta1^{-/-}$) results in embryonic lethality shortly after

Table 1-2: A listing of some of the mutations in adhesion receptors and their ligands leading to human diseases (From Horton MA, 1996)

Receptors	Disease	Phenotype	Ligands	Disease	Phenotype
Integrin $\beta 2$	LADI	Infection	Selectin ligands	LADII	Infection
Integrin $\beta 3$	Glanzmann's syndrome	Bleeding disorder	von Willebrand factor	Haemophilia	Bleeding disorder
Platelet gpIb/IX	Bernard–Soulier syndrome	Bleeding disorder	Coll II	Chondrodysplasia	Skeletal
Integrin $\alpha 2$	—	Mild bleeding disorder	Fibrinogen variants	—	Bleeding, thrombosis
CD43/leukosialin	Wiskott–Aldrich syndrome	Immunodeficiency	Laminin 5	Epidermolysis bullosa	Skin blistering
Integrin $\beta 4$	Epidermolysis bullosa	Skin blistering	Coll I	Osteogenesis imperfecta	Skeletal
CD36	—	No phenotype	Coll I, III, IV	Ehlers–Danlos syndrome	Skin etc.
			Coll VII	Epidermolysis bullosa	Skin blistering

Murine gene knockout experiments have analysed a wide range of receptors and ligands, e.g. (a) fibronectin, integrin $\beta 1$: early embryonic lethal; (b) COL1A1: skeletal abnormalities; (c) integrin $\beta 2$, selectins: liability to infection. The observed phenotypes generally follow those seen with spontaneous mutations in human disease. Many polymorphisms have been discovered in adhesion proteins (e.g. Pl^A in integrin $\beta 3$, Br on integrin $\alpha 2$, Nak on CD36); the majority have no clear phenotype, though mild changes in function can be detected (e.g. fibrinogen) or the variant protein can act as a target for allo- or autoimmunisation (e.g. cadherin homologues in acquired epidermolysis bullosa, platelet integrins in post-transfusion purpura).

blastocyst implantation (Fassler R and Meyer M, 1995; Stephens LE et al, 1995). Recently, Rohwedel et al studied differentiation of $\beta 1^{-/-}$ ES cells via embryoid bodies into skeletal muscle and neuronal cells in vitro to study β1 integrin function during myogenesis and neurogenesis. They found that $\beta 1^{-/-}$ cells showed delayed and reduced myogenic differentiation compared to wildtype and heterozygous ES cells, but acceleration of neuronal differentiation. However, neuronal outgrowth was retarded in the absence of β1 integrins (Rohwedel J et al, 1998). All of this evidence indicates the important roles that integrins play in maintaining physiological functions in vivo.

4. The regulation of integrin function

Integrins on the cell surface can be quantitatively (Ni J et al, 1995; Defilippi P et al, 1991) and functionally (Mould AP, 1996a; Ginsberg MH, 1995) regulated by the cell. In addition, leukocyte adhesion mediated by integrins can be activated by integrin clustering in the plane of the membrane and by changes in integrin cytoskeletal interactions (Lub M et al, 1997; Bennett JS et al, 1999). The regulation of integrin is very important for cells to execute their proper functions, for example, lymphocyte recruitment during inflammation.

4.1 Regulation of expression level of integrin receptors

Some integrins are expressed on normal intestinal leukocytes (e.g. β7) or endothelial cells (e.g. α1, β1) in low amounts. However, upon stimulation, the integrin

expression level is increased (Ni J et al, 1995; Defilippi P et al, 1991). Two mechanisms regulating integrin expression level have been described (i) regulation of integrin expression levels by transcriptional or post-transcriptional mechanisms (Wang D et al., 1995; Zambruno G et al., 1995) and (ii) mobilization of pre-existing intracellular stores (Miller LJ et al., 1987; Molad Y et al., 1994).

Many growth factors and cytokines have been shown to increase integrin expression levels by transcriptional or post-transcriptional mechanisms. These cytokines include TGF-β, EGF, PDGF, IL-4, 6, TNF-β, IFN-γ etc (Kirchberg K et al 1995; Ahlen K et al, 1994; Fujii K et al, 1994). Some of these factors not only increase the expression of integrins that are already present on cells but also initiate de novo synthesis of a particular integrin, i. e. change repertoire (Janat MF et al, 1992). For example, TGF-β increases β1 integrin expression in several cell types by up regulating the levels of mRNAexpression (Wang D et al, 1995; Zambruno G et al, 1995). TGF-β has also been reported to increase the rate of maturation of the β1 subunit (Igontz RA et al, 1987). Therefore, regulation of integrin expression level by this way is not an immediate response.

Some cells can also rapidly mobilize intracellular stores of integrins to their surface upon stimulation. Miller et al reported that when monocytes were stimulated with FMLP or other mediators the cell surface expression of αMβ2 and αXβ2 increased rapidly with a half-maximal time of 2 min. Using detergent permeabilization studies and electron microscopy it was shown that those molecules were held in intracellular vesicles and then mobilized to the cell surface upon stimulation. This phenomenon can also be

found in neutrophils and is thought to be associated with their phagocytosis (Miller LJ et al, 1987). The same phenomenon can also be found in platelets (Niiya K et al, 1987).

4.2 Regulation of functional states of integrin

Regulation of integrin expression levels on the cell surface is not a major aspect of integrin regulation for most cell types. In most situations, cells can regulate integrin functional states without modifying integrin expression levels (Ginsberg MH, 1995; Diamond MS et al., 1994). This allows cells to make rapid responses if necessary.

At least three functional states of integrins have been proposed (Mould AP, 1996a): inactive (I_1) state in which ligand binding sites are hidden, active (I_2) state in which ligand binding sites become exposed, and ligand-occupied (I_3) state. Only in their active state (I_2) can integrins bind to their corresponding ligands. Under physiological conditions integrins can be activated from both the outside (Outside-in) and inside (Inside-out) of the cell.

4.2.1 Activation from inside of the cell

Phorbol esters (Davis GE and Camarillo CW, 1993), chemokines (Campbell JJ et al, 1998) and antibodies to integrin-associated proteins (Brown E et al, 1990) can activate integrins from the inside of the cell. These agents do not interact directly with integrins. Instead, they change integrin functional states through intracellular signal transduction triggered by interacting with other molecules on cell surface (Hynes RO, 1992).

Phorbol esters and integrin cytoplasmic domain phosphorylation: Phorbol esters have been found to induce β2 integrin activation in leukocytes (Valmu L et al, 1991), β1 integrin activation of haemopoietic cells (Shimizzu Y et al, 1990; Stupack DG et al, 1994) and β3 integrin activation of platelets (Parise LV et al, 1990). When lymphocytes are treated with Phorbol esters, homotypic aggregation mediated by αLβ2 is increased. This effect is thought to be mediated by protein kinase C which finally causes the phosphorylation of the cytoplasmic domain of β2 integrins (Valmu L et al, 1991). The α chain is constitutively phosphorylated in leukocytes, but the β subunit only becomes phosphorylated after activation (Hibbs ML et al, 1991). By using purified integrin β2 chains, Valmu L et al have demonstrated that purified protein kinase C could phosphorylate integrin β subunit (Valmu L et al, 1991). Hibbs ML et al further studied the role of the β subunit cytoplasmic domain in cell adhesion. They made mutations in the cytoplasmic portion of the β2 subunit. The mutant β2 subunit was then coexpressed with wild-type αL subunit on COS cells. They found that the 28 amino acids in the N terminus of the cytoplamic domain were necessary for adhesion. Point mutation of the cytoplasmic domain demonstrated that Phe 766 was essential for adhesion. Ser756 was the major phosphorylated residue. Mutation of one or more of the three adjacent threonines Thr758-760 decreased adhesion (Hibbs ML et al., 1991). Using chimeric integrins containing the external domains of platelet integrin IIb/IIIa and the cytoplasmic region from LFA-1 it was demonstrated that phosphorylation of Thr758-760 were important for cytoskeletal binding (Peter K et al., 1995). Recently, Valmu L et al further characterized β2 integrin phosphorylation in phorbol ester-activated T lymphocytes (Valmu L et al, 1999). They found that when T cells were stimulated by phorbol esters,

Ser756 was the major phosphorylation site. However, when the serine/threonine phosphatases inhibitor okadaic acid was added into the system, different phosphopeptide maps were produced. Phosphorylation of two of the threonine residues in the threonine triplet Thr758-760 was detected. Stoichiometric measurements of β2 integrin phosphorylation showed that around 10% of the β2 integrin molecules were phosphorylated upon phorbol ester stimulation of T cells and that the phosporylation increased to 30% of the β2 molecules in the presence of okadaic acid (Valmu L et al, 1999). These results suggest the existence of a strong dynamic phosphorylation in serine and threonine residues of the β2 integrins. Induction of β2 integrin phosphorylation might regulate lymphocyte adhesion by increasing the avidity of β2 integrins through integrin clustering rather than changing the affinity of individual β2 integrin molecule on the cell surface (Lollo BA et al, 1993; Stewart MP et al, 1996). This suggests that increased cell adhesion mediated by integrin to ligand is not always by integrin functional state changes. It is still unknown which kinase(s) phosphorylates the cytoplasmic domain of β subunits in vivo. However, Hannigan GE et al on studying β1 integrin associated-proteins using a yeast two hybridization system have shown that integrin-linked kinase (ILK) might be a candidate since ILK could phosphorylate a peptide derived from the cytoplasmic domain of β1 integrin in vitro. Furthermore, ILK co-purified with β1 integrins (Hannigan GE et al, 1996). Kanner et al further used tyrosine phosphorylation inhibitors to prevent activation of adhesion through CD3. They concluded that tyrosine phosphorylation might be physiologically important, and this reaction probably precedes a protein kinase C step (Kanner SB et al, 1993).

Based on these data, it can be inferred that phorbol esters-triggered protein kinase C pathway may regulate integrin functions on the cells. Activated protein kinase C may trigger the activation of tyrosine or serine/threonine kinases, which lead to the phosphorylation of integrin cytoplasmic domain. ILK may be one of the candidates of the kinases responsible for the integrin cytoplasmic domain phosphorylation. The phosphorylated cytoplasmic domain of integrin may, through the interaction with cytoskeletal components, cause the rearrangement of the cytoskeleton, resulting in avidity changes of the integrin on cell surface.

Integrin-associated proteins and integrin regulation: Antibodies to integrin-associate proteins have also been shown to alter the cell binding ability to immobilized integrin ligands. Examples of the integrin-associated proteins are members of the tetraspanin superfamily, CD19 and CD47 (Brown E et al, 1990; Berditchevski F et al, 1996; Xiao J et al, 1996).

The tetraspanin superfamily has been found to be important integrin-associated proteins. The basic structural motifs in tetraspanins include the presence of four hydrophobic, putative transmembrane domains (TM1-TM4), forming two unequal extracellular loops (EC1 and EC2), with short intracellular amino and carboxyl tails. Some tetraspanins (CD81, CD82, CD9, CD63) are found in virtually all tissues, whereas others are tightly restricted, such as CD37 (B cells) or CD53 (lymphoid and myeloid cells) (Maecker HT et al, 1997). CD81, CD9, CD53, CD63 and CD82 have all been found in association with each other on the cell surface and with certain integrins and HLA-DR antigens in various types of human cells (Rubinstein E et al, 1996). All of these

tetraspanins associate with the β1 integrins α3β1, α4β1 and α6β1 (Behr S and Schriever F, 1995; Berditchevski F et al, 1995; Berditchevski F et al, 1996; Nakamura K et al, 1995; Radford KJ et al, 1996; Rubinstein E et al, 1994). The existence of a tetraspan network has been proposed (Rubinstein E et al, 1996). This network, by connecting several molecules, may organize the distribution of cell surface proteins and play a role in signal transduction, cell adhesion and motility.

Integrin-associated proteins may use different pathways to activate integrins. For example, anti-CD9 mAbs give rise to increased tyrosine phosphorylation of p72syk in platelets (Ozaki Y et al, 1996). Recent evidence also suggests a novel linkage between PI4-kinase, the tetraspanin proteins CD63 and CD81, and α3β1 integrin (Berditchevski F et al 1996; Shaw AR et al 1995). CD47 is a β3 integrin-associated protein. A CD47 agonist peptide (4N1K) derived from the CBD of TS-1 causes αIIbβ3 activation and stimulates platelet aggregation. 4N1K binding selectively induced the phosphorylation of Lyn and Syk and their association with focal adhesion kinase (FAK). Moreover, a complex between CD47 and c-Src has been detected by immunoprecipitation (Schwartz MA et al 1993; Brown E et al 1990).

CD19 receptor has been found to be associated with the β1 family integrin receptors on human B-cell precursors as well as mature B-lymphocytes. The engagement of the β1 family integrin receptors α4β1 and α5β1 with monoclonal antibody homoconjugates leads to rapid activation of the CD19-associated protein-tyrosine kinases and results in hyperphosphorylation of CD19 on tyrosine residues (Xiao J et al, 1996). However, engagement of CD19 on human B lymphocytes can also induce binding of B cells to interfollicular stroma of human tonsils via integrin α4β1 and fibronectin. This

process requires an intact cytoskeleton (Behr S and Schriever F, 1995). CD19 is physically associated with β1 integrin in the immunoprecipitation (Xiao J et al, 1996). The linkage between CD19 and integrin has been demonstrated to be mediated by tetraspans CD9, CD81 and CD82 (Horvath G et al, 1998).

It should be noted that the roles of integrin-associated proteins on integrin regulation are based on the facts that antibodies to integrin-associated proteins can influence integrin-mediated cell functions. However, it is not clear what roles these interactions play under physiological conditions.

Therefore, activation of various integrins can be mediated from within cells. The exact mechanisms are not fully elucidated, but may trigger different signal transduction pathways to cause integrin cytoplasmic domain phosphorylation, which finally results in the changes of integrin functional states.

4.2.2 Activation from outside of the cell.

A variety of extracellular stimulators such as divalent cations, antibodies to integrins, integrin ligands and bifunctional reducing agents such as DTT can activate integrin functions.

Divalent cations and integrin regulation: As discussed above integrins need divalent cations for activity. Depending on the integrins, divalent cations may have different effects on integrin functions. Research from this laboratory and others has

shown that Mn^{++}, Mg^{++} can activate β1 integrin-mediated cell adhesion, while Ca^{++} may have negative effects. Mn^{++} promotes high levels of ligand binding, while Mg^{++} promotes lower levels of binding (Ni HY et al, 1998b).

Part of the I-domain structure in the β2 integrins is the binding site of Mg^{++}. Mould AP et al used purified α5β1 integrin from placenta to study the effects of different combinations of cations on ligand binding. They found that the cation-binding sites within α5β1 were not all identical. Ca^{++} strongly inhibited Mn^{++}-supported ligand binding by noncompetitive mechanism, while Ca^{++} acted as a direct competitive inhibitor of Mg^{++}-supported ligand binding (Mould AP et al. 1995a). These results suggest that the ligand-binding capacity of α5β1 integrin can be regulated in a complex manner through separate classes of binding sites for Mn^{++}, Mg^{++} and Ca^{++}. However, each of these three divalent cations can increase β2 integrin-mediated cell adhesion by different mechanisms. Ca^{++} is able to increase integrin avidity by clustering integrin receptors in the plane of membrane, while Mn^{++} and Mg^{++} increase the affinity of each integrin (Horton MA, 1996). Shimizu Y and Mobley JL characterized the divalent cation requirements for the adhesion of human peripheral CD4+ T cells to four distinct integrin ligands: the α4β1 and α5β1 ligand fibronectin, the α4β1 ligand VCAM-1, the LFA-1 ligand ICAM-1, and the α4β1 bacterial ligand invasin. Distinct divalent cation requirements for T cell adhesion to each of these ligands were found: Mg^{++}/EGTA selectively up-regulated T cell adhesion to ICAM-1. Mn^{++} could up-regulate adhesion to ICAM-1, fibronectin and VCAM-1, while Ca^{++} could selectively support adhesion to VCAM-1 but inhibited Mn^{++}-dependent adhesion to ICAM-1. Binding to invasin is maximal in the presence of Ca^{++}, Mg^{++} or Mn^{++} (Shimizu Y and Mobley JL, 1993). These

results suggest that an individual integrin receptor-ligand interaction can be specifically and selectively regulated by the modification of divalent cations. Divalent cations may bind directly to the divalent cation-binding site of both α and β subunits and I domain of α subunit, and cause a structural change upon activation (Lee OJ et al, 1995). Divalent cation modifications provide only a partial increase in integrin functional activity. The up-regulated integrin function by divalent cation, in many instances, can be increased even further in the presence of additional activation signals such as PMA (Shimizu Y and Mobley JL, 1993). This indicates that integrins can assume multiple levels of activity that are dependent on a combination of the divalent cations that are present and on the activation state of the T cells.

However, the precise role of divalent cations in integrin-ligand interactions is still uncertain. Mould AP proposed that a major role for divalent cations is to directly induce a conformational change in integrins, which is required for exposure of ligand binding sites. Thereby divalent cation could shift a conformational equilibrium between inactive and active states in favour of the active state (Mould AP, 1996a).

Anti-integrin mAbs and integrin regulations: Monoclonal antibodies to integrins can be divided into three groups based on their effects on integrin ligand binding activities: stimulatory, inhibitory and neutral antibodies (Ni HY et al, 1998a, Neelamegham S et al, 1996; Munoz M et al, 1996). Antibodies to different regions of β1 integrins may cause different effects. The activation also depends on the cellular environment and the integrin ligands. Ortlepp et al found that KIM127 mAb recognized CD18 and activated at least CD11a/CD18 and CD11b/CD18 integrins. When K562 cells

were transfected with CD11a/CD18 integrins and treated with this antibody, an increased binding was observed both to ICAM-1 and ICAM-3, whereas another anti-CD18 mAb, KIM185 only stimulated adhesion to ICAM-1. Neither antibody had any effect on binding to coated ICAM-2 (Ortlepp S et al, 1995).

The mechanisms by which these mAbs to integrins regulate integrin functions are not very clear. It seems that for some inhibitory antibodies this regulatory effect is not due to competition with ligand for the ligand binding site on the integrin. Rather, it may relate to conformational changes in integrins. Binding of stimulatory antibodies or inhibitory antibodies to β1 integrins can also cause a change in the expression level of Cation-Ligand-Influenced-Binding-Site (CLIBS) (Ni HY et al, unpublished data). It has also been shown that CD11b or CD18 antibodies induce increased levels of intracellular Ca^{++} which may be an important molecular mechanism of regulation of adhesion (Ng-Sikorski et al., 1991). Based on the model of the modulation of integrin affinity through conformational changes, Mould AP proposed that stimulatory antibodies could be classified into two groups: those binding selectively to the active (I_2) state and those that bind selectively to the ligand-occupied (I_3) conformation. Antibodies preferentially binding to the active state (I_2) or ligand occupied state (I_3) will stabilize this state and shift the conformational equilibrium between inactive and active states or between active state and ligand occupied state in favor of the active state or ligand occupied state, therefore, promoting the ligand binding. Inhibitory antibodies (take mAb 13 for example) bind with much lower affinity to the ligand occupied state than to the unoccupied (I_2) state, and hence destabilize ligand binding by shifting a conformational equilibrium in favor of the unoccupied state (Mould AP, 1996a).

DTT and integrin regulation: Integrins on the cell surface can be activated by reducing agents in several systems. There is an obligate requirement for a bifunctional thiol with a minimal spacing of four carbons between the two -SH groups (Edwards BS et al, 1995). DTT can activate β1, β2 and β3 integrin binding in a number of systems (Edwards BS et al, 1995; Ni HY et al, 1998; Peerschke EI et al, 1995). Davis GE and Camarillo tested the effects of DTT on 120 kD FN binding to purified α5β1 integrin from HL-60 cells and fibroblasts. They found that the addition of DTT increased binding in a dose-dependent manner by three to four fold. There were no differences between the DTT activation of α5β1 integrin from HL-60 or fibroblasts (Davis GE and Camarillo CW, 1993). These results suggest that DTT may induce leukocyte adhesion to FN in part by directly increasing the binding ability of α5β1 for FN. As mentioned before, β subunits contain 56 cysteines which are absolutely conserved within β subunits of the integrin family, ranging from Drosophila to humans (Bogaert T et al, 1987; DeSimone DW et al, 1988). These cysteines are predicted to form 28 nonconsecutive disulfide bridges and the α subunits also contain disulfide bonds (Calvete JJ et al, 1991; Poncz M et al, 1987). One possible mechanism of activation by DTT may be that DTT changes the conformation of α5β1 integrin through the reduction of disulfide bonds with the integrin to facilitate ligand access (Davis GE and Camarillo CW, 1993). This possibility is further supported by results from Gofer-Dadosh N et al (Gofer-Dadosh N et al, 1997). They found that collagen binding to α2β1 integrin induces the formation of a new disulfide bond in α2β1 integrins. All these data reflect the possible roles of disulfide bond exchange in the regulation of integrin functions.

However, in other systems Edwards BS et al found that DTT could dramatically increase αLβ2 integrin mediated adhesion to ICAM-1. This increased cell adhesion was not related to the disulfide bond reduction in αLβ2 integrin induced by DTT because DTT activated αLβ2 on HSB2 T cells had no detectable disulfide reduction in αL or β2 subunits. DTT treatment of NK cells did not hinder binding of mAbs that recognized epitopes in the potentially DTT-susceptible cysteine-rich domain of the β2 chain. Instead, the avidity modulation of αLβ2 by DTT required actin polymerization and was abrogated by the protein kinase C inhibitor calphostin C (Edwards BS et al, 1995). In the research of the effects of DTT on β2 integrin-mediated cell aggregation, Lunam EB et al demonstrated that enhanced aggregation induced by DTT required sustained energy output, suggesting that the effects induced by DTT were intracellular rather than strictly conformational control (Lunam EB et al, 1996).

Thus, DTT can regulate integrin functional states on the cells. The mechanisms of DTT induced integrin functional changes are not very clear. Depending on the integrins, DTT may cause the reduction of disulfide bonds in integrins, resulting in integrin conformational changes which further lead to integrin affinity changes to ligands. On the other hand, DTT may also cause the changes in integrin avidity to ligands by the rearrangement of cytoskeleton or the induction of other signaling events.

Integrin ligands: Integrin ligands include members of immunoglobulin superfamily or extracellular matrix. Data have shown that integrin ligands may be involved in the activation of integrins.

The 22-amino-acid peptide P1 from the first Ig domain of ICAM-2 has been demonstrated to strongly induce T cell aggregation and natural killer cell cytotoxicity. This is mainly mediated by CD11a/CD18-ICAM-1 interaction (Li R et al, 1993). This peptide also strongly stimulates CD11b/CD18-ICAM-1-mediated cell aggregations of the monocytic cell lines THP-1 and U937, and CD11b/CD18 and CD11c/CD18-mediated binding of THP-1 cells to fibrinogen and iC3b coated on plastic (Li R et al, 1995). Similar phenomena have also been observed in integrin-extracellular matrix interactions. Du X et al has demonstrated that a synthetic RGD peptide ligand from fibrinogen which specifically binds to activated αIIbβ3 integrins can induce fibrinogen to bind to αIIbβ3 integrins in platelets (Du X et al, 1991). The mechanisms of P1-induced β2 integrin activation or synthetic RGD peptide ligands induced αIIbβ3 integrin activation are not understood, but presumably peptide binding induces a conformational change in the integrin, exposing binding sites for the ligands, ICAMs or fibrinogen (Li R et al, 1995; Du X et al, 1991).

Collectively, these results indicate that integrin activators may interact directly with integrins to cause integrin conformational changes. This interaction may also trigger signal transduction into cells, which leads to integrin functional changes. Integrins can act as true signaling receptors in variety of cell types, although they lack intrinsic enzymatic activity.

The intracellular signaling pathways triggered by integrins are directed to two major functions: organization of the cytoskeleton and regulation of cellular behavior including differentiation and growth.

4.3. Activated integrins can be clustered to form focal adhesions

Unoccupied or latent integrins are randomly distributed on the cell surface. However, when these receptors on some cells bind to their ligands, for instance fibronectin or other extracellular matrix proteins, they become organized in so-called "focal adhesions" or "focal contacts". In these focal contacts, integrins are clustered on some areas of the cell surface and linked to cytoskeletal elements such as talin, α-actinin, paxillin and focal adhesion kinase (Clark EA and Brugge JS, 1995; Plopper G et al, 1995). Cells use these integrin-clustered areas to adhere to the extracellular matrix (ECM). Multiple receptor-ligand interactions in close proximity are thought to overcome the relatively low affinity of the receptor to its ligand and may result in stable adhesion.

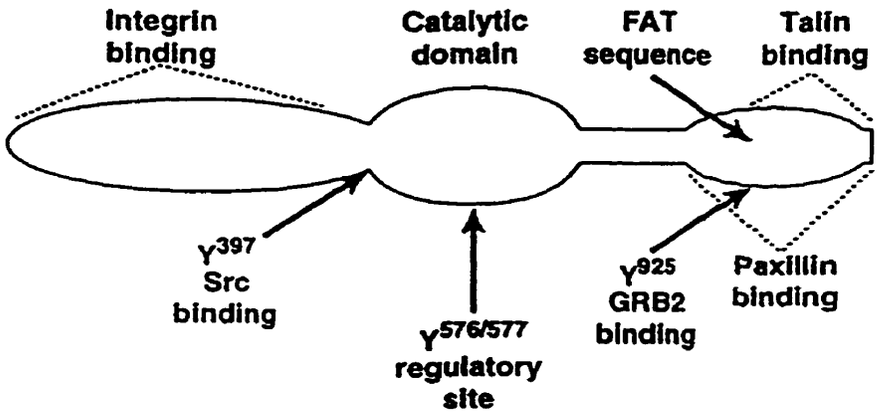
Focal adhesion kinase (FAK) is important in the formation of focal adhesion (Schaller MD and Parsons JT, 1994; Parsons JT, 1996). Focal contacts are areas of close contact between a cell and its extracellular matrix and consist of additional cytoskeletal proteins, adaptor molecules and kinases. It is through this recruitment to focal contacts and the complex protein interactions that integrins are believed to engage the different signal transduction pathways ultimately mediating their physiological effects (O'Toole TE, 1997). Chicurel ME et al used high-resolution in situ hybridization to investigate whether cell binding to the ECM promoted formation of a cytoskeletal microcompartment specialized for translation control at the site of integrin binding. Their

results show that mRNA and ribosomes are rapidly and specifically localized to focal adhesion complexes when cells bind to ECM-coated microbeads. This suggests a new type of gene regulation by integrins (Chicurel ME et al, 1998). Recruitment of integrins and cytoskeletal linker proteins to focal contacts are ligand isoform-specific (Dogic D et al, 1998). For example, fibroblasts adhesing to on laminin 1 ($\alpha 1\beta 1\gamma 1$) form mainly filopodia-like structures. The integrin subunits, cytoskeletal linker proteins including vinculin, talin and paxillin are recruited into thick and short aggregates localized at the termini of actin stress fibers. While on laminin 5 ($\alpha 3\beta 3\gamma 2$) fibroblasts develop lamellipodias and integrin subunits and cytoskeletal linker proteins appear as dots or streaks clustered on a long portion of actin microfilaments (Dogic D et al, 1998).

FAK is reported to be linked to the β subunit of integrins and to colocalize with integrins upon integrin clustering. Integrin activation leads to FAK tyrosine phosphorylation (Hildebrand JD et al., 1993). The mechanism by which integrins activate FAK is unclear. It is proposed that, upon recruitment to focal adhesion complexes by talin, FAK may undergo conformational change and interact through its amino terminal domain with the β subunit tail (Richardson A and Parsons JT, 1996). It is clear that this is tightly coupled to the process of assembly of focal adhesion complexes (Cohen GB et al, 1995). The phosphorylation of FAK is believed to initiate a cascade of phosphorylation events and new protein interactions required for adhesion-dependent signaling complexes (Cohen GB et al, 1995; Heldin CH et al, 1995; Pawson R et al, 1995).

FAK is broadly expressed in most cell lines and tissues (Schaller MD and Parsons JT, 1994). The amino acid sequence of FAK is highly conserved among humans and

A.


Integrin binding
Catalytic domain
FAT sequence
Talin binding
Y397 Src binding
Y576/577 regulatory site
Y925 GRB2 binding
Paxillin binding

B.

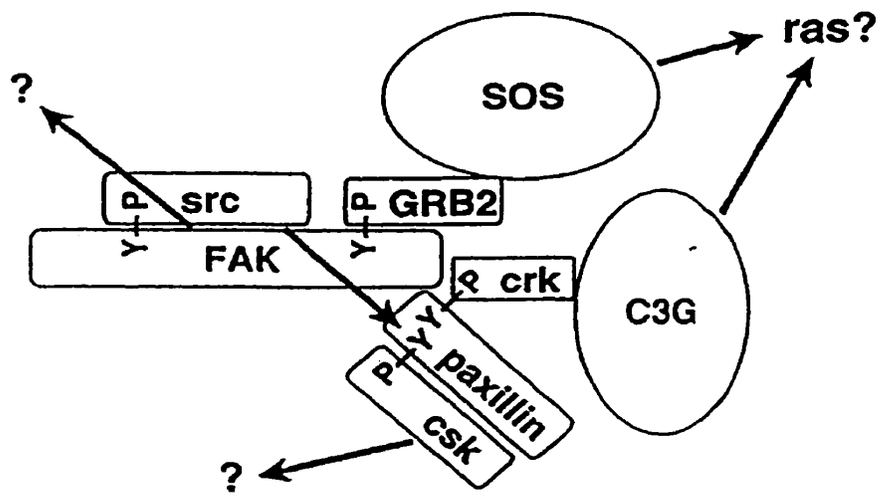

?
SOS
ras?
P
Y
src
FAK
P
Y
GRB2
P
crk
C3G
Y
Y
P
paxillin
csk
?

Figure 1-5: (A) Structure of FAK. The domains of FAK and the sites of interaction with binding proteins are shown. Tyrosine residues (Y) that can be phosphorylated and the SH_2 proteins to which they bind are also shown. (B) FAK complexes. FAK can form complexes with a number of signaling proteins. Arrows represent potential signaling pathways that may be activated by these proteins (Schwartz MA et al, 1995).

other species. The molecule is composed of a central kinase catalytic domain flanked by large amino terminal and carboxyl terminal sequences (Hanks SK and Polte TR, 1997).

FAK can form complexes with a number of signaling proteins (Figure 1-5). FAK has six phosphorylation sites (Tyr 397, 407, 576, 577, 861 and 925) (Hanks SK and Polte TR, 1997). The amino acid residues 31-376 have been demonstrated to interact with the cytoplasmic domains of β1, β2 and β3 integrins in vitro (Schaller MD and Parsons JT, 1995). The autophosphorylation at site Tyr397 generates a high-affinity site for the binding of the Src family of nonreceptor kinases via an SH_2 domain. Src interaction with FAK causes the phosphorylation of Tyr407, 576, 577 and 861, causing the maximal activation of FAK (Brown MT and Cooper JA, 1996). The carboxyterminal domain is characterized by two proline-rich domains (PR1: 712-715 and PR2: 873-876) that may be responsible for binding SH_3 domain-containing proteins such as P85 of PI-3 kinase (Guinebault C et al., 1995; Polte TR et al., 1995), Cas and Graf (Hanks SD and Polte TR, 1997). The Tyr-925 is the binding site for SH_2 domain of Grb-2 (Schlaepfer DD et al., 1994) which leads to activation of the ras pathway. The focal adhesion targeting (FAT) sequence is responsible for the localization of FAK to the focal adhesions. FAT is also the binding site for talin, paxillin which connect to cytoskeleton (Schaller MD et al., 1997). Collectively, these results suggest that FAK seems to play a pivotal role in integrin signal transduction and the regulation of cytoskeletal organization.

4.4 Integrin cytoplasmic domain is associated with cytoskeleton

The integrin cytoplasmic domain can associate with the cytoskeleton (Chicurel ME et al, 1998). Actin filaments are anchored at the plasma membrane in focal contacts,

which in addition to the integrins contains various cytoplasmic proteins, e.g., vinculin, talin and α-actinin (Dogic D et al, 1998). It is believed that integrins bind via their β subunits to cytoskeletal components (Hynes RO, 1992). Studies involving mutant β1 subunits, containing cytoplasmic deletions and point mutations have led to the identification of nine amino acids clustered in three regions (cyto-1, 2 and 3) that contribute to focal adhesion formation (Reszka AA et al, 1992). Binding sites of actin-binding protein filamin have also been identified (Sharma CP et al, 1995).

Phosphorylation has been shown to play a role in the interaction of integrins with specific cytoplasmic components. The cytoskeletal component talin, vinculin and paxillin have been reported to be phosphorylated in cells when integrins are activated (Leventhal PS and Feldman EL, 1996). Furthermore, on lymphocytes treated with PMA, talin co-distributes with integrins capped by antibodies (Burn P et al. 1988). These data suggest a role of cytoskeletal components in the regulation of integrin functions. However, the details of the mechanisms by which cytoskeletal components regulate integrin function are not clear. Jone SL et al, in their research on L-plastin phosphorylation in integrin activation, found that, when introduced into the cytosol of freshly isolated primary human PMN and monocytes, L-plastin-derived peptides containing the phosphorylation site (Ser5) rapidly induced leukocyte integrin-mediated adhesion. Ser5 to Ala5 mutation abolished the ability of the peptide to induce adhesion (Jones SL et al, 1998). These data suggest a role of L-plastin in the regulation of leukocyte adhesion. Lub M et al reported that filamentous actin could both enhance and inhibit LFA-1-mediated adhesion, depending on the distribution of LFA-1 on the cell surface. Disruption of actin cytoskeleton in resting lymphocytes causes increased binding of LFA-1 to ICAM-1,

while in lymphocytes with clustered LFA-1 on the cell surface because of the treatment with IL-2 or phytohemagglutinin, strong adhesion to ICAM-1 mediated by LFA-1 is inhibited by disruption of the actin cytoskeleton (Lub M et al, 1997). Thus, it can be inferred that cytoskeletal elements may prevent movement of LFA-1 over the cell surface, resulting in inhibiting clustering and strong ligand binding. When released from cytoskeletal elements, LFA-1 can move laterally, leading to ligand binding. Subsequent outside-in signals then stimulates actin polymerization which stabilizes cell adhesion (Lub M et al, 1997). A similar observation was made by Bennett JS et al. They found that the actin cytoskeleton in unstimulated platelets constrained αIIβ3 in a low affinity state. Agonist-stimulation increases platelet cytosolic calcium and initiates actin filament turnover, which then relieves cytoskeletal constraints on αIIbβ3, allowing it to assume the high affinity conformation required for soluble ligand binding (Bennett JS et al, 1999).

4.5 Cytoplasmic regions of integrin are involved in the regulation of integrin functions

It has been proposed that integrin affinity states may be ultimately regulated by the cytoplasmic regions of the integrin (Hynes RO, 1992).

Integrin β cytoplasmic domains mediate integrin function, presumably by binding to specific cytoskeletal and signal proteins. Chimeric receptors containing β cytoplasmic domains connected to extracellular domain of interleukin-2 receptor or CD4 extracellular domain have been shown to be sufficient to activate specific signaling events (Akiyama SK et al, 1994; Lukashev ME et al, 1994). Two key regions have been identified in the β subunit. The first is a highly conserved NPXY motif located in the cytoplasmic domains

of β1-β7, excluding β4 (O'Toole TE et al, 1995; Filardo EJ et al, 1995). Mutations within this conserved motif in the β1 and β3 cytoplasmic domains inhibit the ability of β1 and β3 integrins to localize to focal adhesions (Sastry SK and Horwitz AF, 1993; Ylanne J et al, 1995). Using the yeast two-hybrid system, ICAP-1 (integrin cytoplasmic domain associated protein-1) was shown to specifically interact with the NPXY region of β1 cytoplasmic domain (Chang DD et al, 1997). At present the biological role of ICAP-1-integrin interaction is unknown. However, ICAP-1α, an isoform of ICAP-1 expressed in most cells, is a phosphoprotein and the extent of its phosphorylation is found to be regulated by the cell-matrix interaction (Chang DD et al, 1997), suggesting an important role of ICAP-1 during integrin-dependent cell adhesion. The second region consists of a highly conserved membrane proximal sequence, KLLXXXXD. Deletion of Leu717-Asp723 within this region in β3 integrins results in constitutive activation (Hughes PE et al, 1995). The terminal aspartic acid residue in β cytoplasmic tails is also necessary for regulation of integrin activation. The terminal residues of the α and β cytoplasmic domains may form a salt bridge which stabilizes an inactive conformation (Hughes PE et al, 1996). The cytoplasmic domain of β4 subunit contains more than 1000 amino acid residues. The two pairs of fibronectin type III repeats have been demonstrated to interact with hemidesmosomes (Spinardi L et al, 1993). All of these data indicate a role of the β cytoplasmic domain in the regulation of integrin functions.

α cytoplasmic domains also play diverse roles in integrin functional regulations. α cytoplasmic domains differ significantly among integrins in their requirements for integrin-ligand interactions. There is evidence from a number of studies that the cytoplasmic domains of α subunits are required for the physiological activation of

integrins. The deletion of sequences C-terminal to the GFFKR motif in the α2, α4 and α6 cytoplasmic domains inhibit cell adhesion (Chan BM et al, 1992; Kassner PD and Hemler ME, 1993; Kawaguchi S and Hemler ME 1993). A truncated α6 integrin with cytoplasmic domain sequence deleted after the GFFKR exhibits impaired adhesion to laminin when it is coexpressed with β1 integrin (Shaw LM and Mercurio AM, 1993). However, in other systems, α cytoplasmic domains are reported not to be required for α5β1 and αLβ2 integrin-mediated cell adhesion (Sastry and Horwitz, 1993). α2, α4 and α5 subunit cytoplasmic domains have no obvious shared sequence motif. Deletion of the cytoplasmic domains from α2 or α4 or α6 causes loss of cell adhesive activity (Kassner PD and Hemler ME, 1993; Kawaguchi S and Hemler ME, 1993; Shaw LM and Mercurio AM, 1993). However, exchange of the α2 and α4 cytoplasmic sequence with the α2, α4 and α5 sequences does not alter the levels of integrin-mediated adhesion (Kassner PD and Hemler ME, 1993; Kawaguchi S and Hemler ME, 1993). It is not clear how these cytoplasmic domains make similar contributions to cell adhesion.

Taken together, it appears that both integrin cytoplasmic tails are associated with intracellular signaling pathways, resulting in changes in the conformation or spatial relationships of the α and β subunits. These effects may be mediated by the binding of activating factors to the integrin tail, or by the removal of a repressor, or by the direct chemical modification of the cytoplasmic domains themselves (Ginsberg MH et al, 1992).

4.6 Integrin cross talk

Integrin crosstalk has been demonstrated in numerous primary cell types and cell lines including macrophages, T cells, smooth muscle cells, neutrophils, monocytes, umbilical vein endothelial cells, CHO cells, K562 cells (Gresham HD et al, 1989; Pacifici RJ et al, 1994; Porter JC and Hogg N, 1997; Simon KO et al, 1997). Integrin crosstalk refers to the phenomena in which the activation of one kind of integrin influences the activities of the other integrin species on the cells. Crosstalk may be initiated by transducing integrins belonging to the β1, β2, or β3 family with targets in any of these families as well (Monier-Gavelle F et al., 1997; Porter JC and Hogg N, 1997). For example, the interaction of the β2 integrin (LFA-1) with its ligand ICAM-1 decreases adhesion mediated by α4β1 and, to a lesser extent, α5β1 integrins (Porter JC and Hogg N, 1997). Signals from integrin α5β1 are necessary for integrin αvβ3 to internalize vitronectin and ligation of the integrin αvβ3 inhibits both phagocytosis and migration mediated by α5β1 on the same cell (Blyston SD et al, 1994, Blystone SD et al, 1995). The β3 cytoplasmic tail is necessary and sufficient for this regulation of α5β1(Blystone SD, 1999). However, the mechanisms responsible for integrin cross talk are still unknown.

In summary, cell adhesion to extracellular matrix or cell-cell aggregation is a complex process. It requires that cells have sufficient amount of adhesion receptors and that the adhesion receptors on the cells must become activated. Lymphocytes are particularly useful for adhesion studies because integrins on lymphocytes are constantly switching from one functional state to another. They attach to the ligands for a finite time

under certain conditions, and then in most cases detach. Studies of these cells may have general applicability. Lymphocytes regulate their adhesive state mainly through integrin functional regulation on their surface. Integrin functional regulation can be achieved by "inside-out" or "outside-in" pathways. Regardless of which pathway cells use in integrin functional regulation, the integrin cytoplasmic domain is absolutely necessary. The cytoplasmic domain is not only involved in signal transduction but also physically connects integrin to cytoskeleton. These interactions between the integrin cytoplasmic domain and cytoskeleton can alter integrin receptor organization on cell surface.

5 Integrin functional regulation and integrin conformational changes

The activation of integrin appears to involve an increase in affinity of the receptor for ligand. These activated receptors may display new epitopes (Ni HY et al, 1998a; Luque A et al, 1996) as a result of conformational changes. Although there is some controversy as to whether integrin activation in the absence of ligand binding is accompanied by large conformational changes, there does appear to have data to support an association of conformational change with activation.

Several studies have shown that integrin regulation is accompanied by integrin conformational changes. These conformational changes are not restricted to the ligand-binding site, but can also occur in other regions (Mould AP, 1995b; Tozer EC et al, 1996). Mould AP proposed a model of the modulation of integrin affinity through conformational changes (Mould AP, 1995a). In this model, ligand binding sites are hidden in the inactive (I_1) state but become exposed in the active (I_2) state upon

stimulation. Recognition of ligand by the active integrin causes further conformational changes, including the increased exposure of cation and ligand-induced binding site (CLIBS) epitope and the reduced exposure of ligand-attenuated binding sites (LABS) epitopes. This model elucidates the relationship between integrin functional regulation and integrin conformational changes. However, the details of the processes are still unclear.

Others have argued that the changes in integrin affinity and conformation are probably overemphasized (Bazzoni G and Hemler E, 1998). Bazzoni G and Hemler E challenged the assumption that integrin activation is accompanied by major conformational changes with the following arguments: (1) There are now numerous examples in which regulation of integrin-dependent cell adhesion may not involve altered ligand-binding affinity (Jakubowski A et al, 1995; Weber C et al, 1996). For example, upon stimulation by chemokines, the binding ability of $\alpha 4\beta 1$ integrin to soluble ligand is not increased. The mechanisms by which chemokine induced increased adhesion of $\alpha 4\beta 1$ integrin to immobilized ligand may involve actin cytoskeleton dependent pathways (Weber C et al, 1996). (2) Reports of various activation-specific anti-integrin antibodies have led to the erroneous idea that physiologically relevant integrin activation is always accompanied by extensive conformational changes. In fact, monoclonal antibodies to integrin neoepitopes can be separated into two categories: (i) ligand-mimetic antibodies and (ii) anti-CLIBS antibodies. Ligand-mimetic antibodies can resemble ligands that bind to integrins. However, since some of the ligand-mimetic antibodies may have higher affinity than ligands, these antibodies are obviously not suitable for detecting additional conformational changes that may accompany increased ligand-binding potential.

6. Evidence for possible roles of long range disulphide bonds in the regulation of integrin function

Evidence has been accumulated to indicate the possible roles of long range disulphide bonds in the regulation of integrin function. (1) Integrins are predicted to have two pairs of long range disulphide bonds that link distant regions of the β chain (Calvete JJ et al, 1991, Wand R et al, 1997). (2) As discussed previously, bifunctional reducing agents with distinct structural requirements (such as DTT) can activate β1 (Ni HY et al, 1998), β2 (Edwards BS et al, 1995) and β3 (Peerschke EI et al, 1995) integrin function on cells. Davis GE and Camarillo CW further demonstrated that DTT could increase soluble 120 kD FN fragment binding to purified α5β1 integrin, indicating that DTT acted in part by directly influencing the integrin-ligand interaction (Daivs GE and Camarillo CW, 1993). DTT might change the conformation of α5β1 integrin through disulfide bond reduction in a way that favors FN binding. (3) Previous data from our laboratory demonstrated that activation of β1 integrin by DTT, Mn++ and RGD peptide is accompanied by an increased exposure of epitopes recognized by mAb B44 and (in the case of DTT and Mn++) N29 but not the other epitopes so far tested in this laboratory (Ni HY et al, 1998a). N29 and B44 epitopes have been localized between amino acid residues 15-54 and 355-425, regions that are close to the two predicted large loops formed by the connection between Cys7-Cys444 and Cys415-Cys671 respectively in the extracellular domain of β1 integrins (Ni HY et al 1998a; Ni HY et al unpublished data). This data could suggest that β1 integrin activation is accompanied by conformational changes around these two disulfide bond pairs. Furthermore, the free –SH groups in integrin β1 subunit on DTT treated Jurkat cells and IM9 cells is significantly increased compared

with untreated cells although there is no data to prove that this may be related to the DTT effects on cell adhesion (Ni HY et al, unpublished data). These phenomena have also been observed in β3 integrins. Honda S et al reported that αIIbβ3 integrin activation by integrin ligands caused exposure of epitope located between residues 1-6 region and between residues 602-690 in β3 subunits. These global changes are thought to be facilitated by long range disulfide bonds between Cys5-Cys435and Cys406-Cys655 in β3 subunits. These regions are also sensitive to divalent cations (Honda S et al, 1995). (4) In addition, Gofer-Dadosh N et al, in the research of ligand-induced change in the affinity of platelet α2β1 integrin to collagen, found that platelets that bound to collagen by α2β1 integrin could be eluted with DTT and 2% SDS but not EDTA, chaotropic agents or low pH. However, DTT followed by acetic acid could also elute platelets. They concluded that (i) ligand binding induces the formation of new intrareceptor disulfide bonds in α2β1 integrin (not receptor-ligand disulfide bridge because type I collagen used in the study contains no cysteines), (ii) this change increases the affinity of the receptor to its ligand (Gofer-Dadosh N et al, 1997).

Based on these data a possible mechanism of integrin activation by integrin activators such as DTT is that these activators can reduce disulfide bonds within the integrin. These could possibly involve disulfide exchange to facilitate ligand binding. Thus, disulfide exchange might be involved in the regulation of integrin function. However, for such a scheme to be plausible this would require the presence of an extracellular disulphide exchange system.

7. Protein disulfide bond formation/exchange is regulated by protein disulfide isomerase

The enzyme protein disulfide isomerase (PDI) is the predominent mediator of intracellular disulfide exchange (Luz JM et al, 1996; Freedman RB et al, 1994). This enzyme is largely retained in the endoplasmic reticulum (Luz JM et al, 1996). However, plasma membrane-associated PDI has also been described (Tager M et al, 1997; Paydas S et al, 1995, Essex DX, 1999). PDI has been shown to be located on the membrane of mature human lymphocytes (Kroning H et al, 1994; Paydas S et al, 1995). A link between PDI and -SH expression level on B cell chronic lymphocytic leukemia (B-CLL) has been demonstrated (Tager M et al., 1997), suggesting a functional relationship between the two. Surface associated thiol disulphide transferase activity has been shown to be necessary for diphtheria toxin activation and for the infection of lymphoid cells with HIV (Mandel R et al, 1993; Hugues JPR et al, 1994).

The role of cell surface PDI in integrin regulation is still unknown. Evidence has shown that activation of platelet adhesion can lead to a marked increase in PDI expression (Chen K et al, 1992). Protein disulphide isomerase mediates platelet aggregation and secretion and it activates GPIIbIIIa (Essex DX, 1999). PDI is demonstrated, in vitro, to catalyze refolding of disulfide-containing proteins (Pigiet VP and Schuster BJ, 1986). Furthermore, PDI in the endoplasmic reticulum can also function as a chaperon protein which is important for the formation of proper protein conformation (Luz JM, 1996). All of this evidence suggests the possible role of PDI in their regulation of integrin function.

PDI regulates protein disulfide bond formation and exchange by the following three reactions: (1) PDI oxidizes cysteine sulfhydryl groups of the protein to form a PDI-protein intermediate complex. (2) The disulfide bonds between PDI and protein are then reduced and PDI is released from the intermediate complex. (3) The isomerization of disulfide bonds is a composite of the reduction step followed by the oxidation step (Figure 1-6A) (Luz JM et al, 1996). PDI is expressed on variety of cell types including lymphocytes and is rich in the endoplasmic reticulum (Noiva R and Lennarz WJ, 1992). The protein is about 57 kD in molecular weight and has two specific domains (CGHC) with homology to the bacterial protein thioredoxin (CGPC). The CXYC motif is the thiol-disulfide interchange catalytic domain. PDI is composed of (Figure 1-6B) (1) a putative signal sequence at its N-terminus which directs PDI to be translocated into the lumen of the ER. (2) ER retention signal containing KDEL or HDEL C-terminus. (3) Two thioredoxin-like domains (CGHC). (4) Two pairs of internal repeats. (5) A peptide binding site (Luz JM et al., 1996; Freedman RB et al., 1994).

Besides its regulation of protein disulfide bond, PDI is a multifunctional protein. It has been reported that PDI can function as a molecular chaperone. PDI may also play a role in the transcriptional activation of interferon-inducible genes (Noiva R and Lennarz WJ, 1992).

The regulatory functions of PDI on the protein disulfide bond can be inhibited by a variety of inhibitors including sulfhydryl blockers such as dithiobisnitrobenzoic acid (DTNB) (Couet J et al, 1996), the small peptide inhibitors, somatostatin and tocinoic acid, and bacitracin (Essex DW et al, 1995).

A.

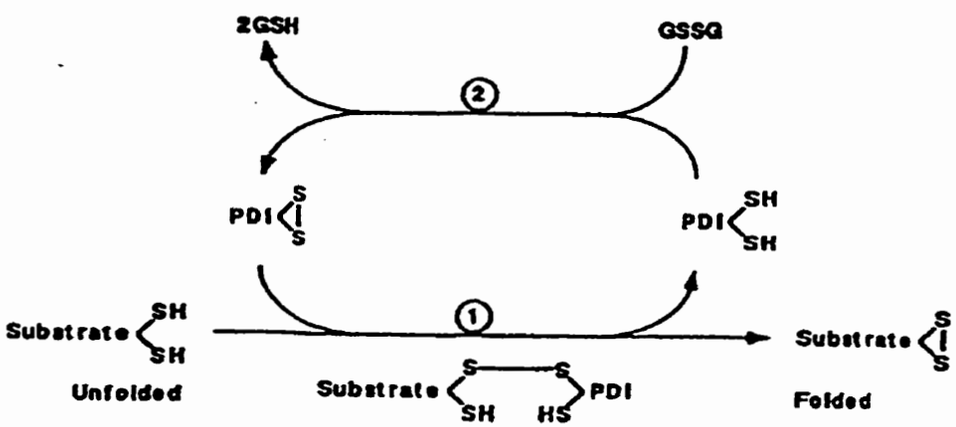

2GSH
GSSG
2
PDI
S
S
PDI
SH
SH
Substrate
SH
SH
1
Substrate
S
S
Unfolded
Substrate
S
S
SH
HS
PDI
Folded

Figure 1-6A: (A) Catalytic activity of PDI (From Luz JM et al., 1996). PDI, with its active sites in disulfide form, can oxidize the sulfhydryls of a substrate to form the disulfide-bonded protein (reaction 1). Presumably in concert with this process the unfolded protein assumes its native state. As shown, an intermediate in the process is presumed to be a mixed disulfide between PDI and the substrate. Recycling of PDI requires its reoxidation by an oxidizing agent such as oxidized GSH (GSSG) as shown in reaction 2.

B.

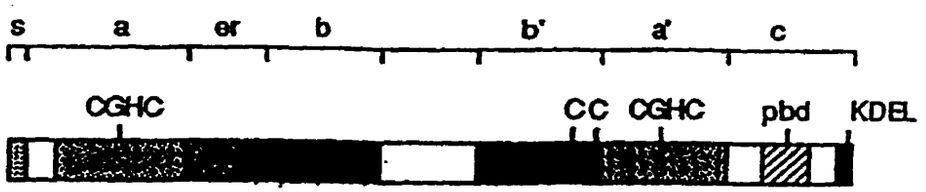


Figure 1-6B: (B) Schematic representation of Human PDI (From Luz JM, 1996). Symbols: s signal sequence, a, a' internally homologous regions with sequence homology to thioredoxin, b, b' internally homologous regions, c highly acidic C-terminal region of the poly-peptide, er region with homology to the estrogen receptor. The active site sequences (CGHC), additional cysteine residues (C), the peptide-binding domain (pbd) and the C-terminal ER retention signal (KDEL) are also indicated.

The membrane-impermeant thiol reagent, DTNB, is a sulfhydryl blocker. It can non-specifically abolish the reductive cleavage of a surface bound disulfide conjugate (Feener EP et al, 1990). DTNB has been widely used in various systems to interfere with surface bound disulfide bonds. It has been reported that DTNB is able to reduce activation of receptor-bound diphtheria toxin (DT) in CHO cells (Ryser HJP et al, 1991). It is also capable of inhibiting infection of human lymphoid cells by membrane-bound HIV (Mandel R et al, 1993). Ryser HJ et al found that, in the presence of 2.5 mM of DTNB, almost 100% of HIV infection to CD4 T lymphocytic cell line H9 was inhibited, while inhibitory monoclonal antibody to PDI, HP13 which inhibits 50% of PDI activity even at a 10- to 15- fold molar excess of mAb over antigen, only caused 83% inhibition of HIV infection. Therefore, it is assumed that the critical sulfhydryls blocked by DTNB include the two cysteine thiols present at both active sites of PDI (Ryser HJ et al, 1994).

Both somatostatin and tocinoic acid are small peptides with two Cys residues. PDI has a common chaperone function. Its peptide-binding site in the C-terminal region and b' domain (see Figure 1-6B) can nonspecifically bind to peptides including somatostatin and tocinoic acid (Klappa P et al, 1997; Klappa P et al, 1998). The interaction between PDI and the bound peptide is further enhanced by the formation of mixed disulfide bonds (Klappa P et al, 1997). It has been demonstrated that the binding of somatostatin inhibited PDI-dependent insulin degradation (Morjana N and Gilbert H, 1991), suggesting that somatostatin inhibits PDI activity by interference with the substrate-binding site in PDI.

A peptide antibiotic Bacitracin is also a potent PDI inhibitor (Essex DW et al, 1995; Mandel R et al, 1993; Hugues JPR et al, 1994). Commericial Bacitracin is

produced by the action of strains of *Bacillus subtilis* and *Bacillus licheniformis*. Bacitracin is a compound composed of a peptide and a non-peptide side chain. The mechanisms by which bacitracin inhibits PDI activity are unclear. Bacitracin contains neither an active group that can block free sulfhydryls nor Cys residue. Therefore, bacitracin may act by a different way to inhibit PDI activity.

Purpose of the project

The present studies were designed to test the hypothesis that disulfide exchange is involved in the physiological regulation of integrin function.

Materials and Methods:

1. Materials:

1.1 Reagents:

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. Media, fetal bovine serum (FBS) and fibronectin 40 kD, 120 kD fragments were obtained from Gibco BRL Life Technologies, Inc. The monoclonal antibody to PDI (clone RL77) (Mandel R, et al, 1993) and monoclonal antibodies (mAb) to human β1 integrin JB1A (Caixia S. et al, 1991), B3B11, N29 (Wilkins JA. et al, 1995), 3S3 (Gao JX. et al, 1995), B44 (Wilkins JA. et al, 1995), HUTS4 and HUTS21 (Luque A et al, 1996), and 15/7 (Bazzoni G, et al, 1998) have been described elsewhere. Monoclonal antibody against human αvβ3 integrins LM609, purified human plasma fibronectin and FITC conjugated goat anti-mouse IgG were obtained from Chemicon Intl., Temecula CA. Recombinant VCAM-1 and CS1 were kind gifts from Dr. D. Staunton (ICOS Corp) and Dr J. McCarthy (University of Minnesota) respectively. HPLC-purified bacitracin A was obtained from Dr. L. Kesner (Department of Biochemistry, Brooklyn, NY).

1.2 Cell lines:

All the cell lines except human peripheral blood mononuclear cells (PBMCs) were obtained from American Type Culture Collection (ATCC).

2. Methods

2.1 Cell culture:

PBMCs were isolated from normal volunteers on Ficoll hypaque according to the manufacturer (Sigma data sheet). Briefly, anti-coagulated venous blood was diluted with equal volume of 0.9% NaCl. 25 ml of diluted blood was then carefully layered into the top of 12.5 ml Ficoll hypaque pre-warmed to room temperature in 50 ml conical centrifuge tube. After centrifugation at 1400 rpm for 20 min, the upper layer was carefully aspirate with a Pasteur pipette to within 0.5 cm of the opaque interface which containing PBMCs. The opaque interface was then carefully transferred into a 50 ml clean conical centrifuge tube. After being washed twice with 30 ml phosphate Buffered Saline Solution, the isolated PBMCs were cultured at 37^0C for 3 days in RPMI 1640 containing 10% FBS and 10 μg/ml of PHA-P. Recombinant human IL-2 (25 units/ml) was added. Cells were maintained by half dilution every 2-3 days in fresh media containing IL-2 until day 12. These cells were used for the experiments. PBMCs were re-stimulated every 14 days by freshly isolated PBMCs which were irradiated at 2500 rad in 1 : 5-10 (PBMCs : irradiated cells) ratio in the presence of 10 μg/ml of PHA-P. Recombinant human IL-2 (25 units/ml) was added after 24 hours, and the cells were then maintained in the fresh media containing IL-2 as mentioned above.

All other cells were cultured in RPMI 1640 with 10% FBS, and were maintained by 1 to 10 dilution every 2-3 days until being used in the experiments.

All cells were incubated at 37^0C with 5% CO_2.

2.2 Cell binding assay:

Cells were washed twice in RPMI-1640 without FBS and resuspended in the same media. $2X10^5$ cells were added into each microtiter well coated with 1 μg/well 40 kD or 120 kD fibronectin fragments or laminin or 0.2 μg/well VCAM-1. The plates were incubated at 37^0C for 1 hour. To remove unbound cells, the wells were filled with 0.15 M saline and sealed, the plates were inverted and centrifuged at 70Xg for 5 min. The supernatant was discarded and the adherent cells were fixed and stained with 0.5% crystal violet in 30% methanol for at least 30 min. The plates were washed with tap water and the bound dye was dissolved in methanol. The absorbance was determined at 550 nm.

For induction of cell binding, cells were incubated with 150 ng/ml of anti-CD3 monoclonal antibody at 4^0C for 30 min, and were then further incubated with 1 μg/ml rabbit anti-mouse secondary polyclonal antibody at 4^0C for 1 hour. In PMA or anti-integrin mAb stimulated cell binding assay, cells were incubated with 50 ng/ml PMA at room temperature for 5 min or 10 μg/ml stimulatory anti-integrin monoclonal antibody at 37^0C for 30 min. These cells were then added into each microtiter well coated with integrin ligands for cell binding assay.

For cell binding inhibition test, cells were treated with the indicated concentration of inhibitor at 37^0C for 30 min prior to addition to assay for their adhesive potential. Inhibitor was retained in the cell suspension during the assay unless indicated.

2.3 Flow cytometry analysis:

Cells were washed twice in RPMI, pre-incubated with the indicated reagents at 37°C for 30 min in the presence or absence of 50 mM EDTA, followed by incubation with 5 μg/ml of the indicated mAb to β1 integrin at 37°C for 30 min. Cells were washed twice in PBS and were then incubated with FITC-conjugated goat anti-mouse immunoglobulin at 4°C for 1 hour. Fluorescence analysis was performed with a BD FACScaliber.

2.4 Purification of α5β1 and αvβ3 integrin from placenta:

Human placenta was homogenized in 25 mM Tris-HCl pH 7.6 containing 150 mM NaCl, 2 mM $CaCl_2$, 50 mM n-octylglucopyranoside and 1 mM PMSF. The precipitate was removed by spinning at 10,000Xg for 1 hour at 4°C.

For purification of α5β1 integrins, the supernatant was sequentially passed through an ovalbumin Sepharose 4B and a JB1A (anti-β1 integrins) Sepharose 4B column at a rate of 1 ml/min. The column was sequentially washed with 20 column volumes of each of 1) 0.1% NP-40 in 25 mM Tris-HCl pH7.6 containing 150 mM NaCl, 2 mM $CaCl_2$, 2) 0.1% NP-40 in 0.01 M sodium acetate buffer pH4.5. Beta-1 integrin was eluted by 10 mM sodium acetate buffer pH 3.6 which contained 0.1% NP-40 and neutralized immediately with 3 M Tris-HCl pH 8.8. The purified β1 integrins were further separated on an anti-α5 integrin JBS5 affinity column. Purified α5β1 integrin were recovered in elution buffer (10 mM NaAc, pH 3.5, 100 mM $CaCl_2$, 100 mM $MgCl_2$ and 50 mM n-Octyl-β-D-glucopyranoside) and immediately neutralized by 1 M Tris-HCl, pH 8.0.

For the purification of αvβ3 integrin, the supernatant was incubated with covalently-linked anti-αvβ3 monoclonal antibody (LM609) beads at 4^0C overnight. Following the extensive washing of the beads with buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.1% NP40), αvβ3 integrins were eluted with buffer containing 100 mM glycin-HCl, pH 2, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM n-Octyl β-D-Galactopyranoside. The elute was immediately neutralized with 2 M Tris-HCl, pH 8.2.

2.5 Biotin labeling of proteins:

Proteins were dialyzed in 100 mM HEPES pH 8.5. The concentration of the proteins was adjusted 10 mg/ml then Biotin N-Hydroxysuccinimide ester (BNHS) (Pierce product) was added at 1/10 the weight of the proteins. The protein solution was incubated at room temperature for 2 hours. The reaction was terminated by adding 5 µl of ethanolamine. The biotinylated protein was then fully dialyzed against PBS.

2.6 Protein quantification:

Protein quantification was performed according to the protocol of BCA protein assay kit from Pierce. Briefly, 10 µl of standard or unknown sample was pipeted into the wells of 96-well plate, then 200 µl of the working reagent was added to each well and mixed. The plate was incubated at 37^0C for 30 min. After cooling the plate to room temperature, absorbance at 550 nm was determined.

2.7 Ligand binding to purified β1 integrin:

Microtiter wells were coated with purified α5β1 integrin 50-100 ng/well in dilution buffer (25mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Ca^{++} and 0.5 mM Mg^{++}) at 4^0C overnight. After washing with washing buffer (dilution buffer with Mg++ and Ca^{++} but containing 1 mM Mn^{++}, 1% heat denatured FCS, 0.05% Tween-20), the plates were blocked with 10% heat denatured FCS in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM Mn^{++}. The indicated amount of biotin-labeled fibronectin diluted in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM Mn^{++} and 1% heat denatured FCS was added in the presence or absence of indicated amount of bacitracin. The plates were incubated at 30^0C for 3 hours and subsequently washed 5 times with washing buffer. Alkaline phosphatase conjugated avidin was added and the plates were then incubated at 37^0C for 30 min. The plates were washed 5 times and the colour was developed by adding substrate and incubation at 37^0C for 30 min. O.D value was determined at 405 nm.

2.8 SDS-Polyacrlamide Gel Electrophoresis

8% polyacrylamide gel electrophoresis under reducing conditions was performed. The gel was stained by 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid overnight, and was destained by successive incubation in destaining solution containing 5% methanol and 7.5% acetic acid.

Results

1. Effects of PDI inhibitors on β1 integrin-mediated T lymphocyte adherence

Bacitracin, tocinoic acid, somatostatin, dithiobisnitrobenzoic acid (DTNB) and anti-PDI monoclonal antibody (RL 77) have previously been shown to be potent inhibitors of cell surface PDI or thiol-reductase (Essex DW, 1995). Adhesion of IL-2 dependent T cells to fibronectin was mediated mainly by α4β1 and α5β1 integrins (Bacon KB, 1995). Cells treated with 5 mM DTNB (Figure 2-1A), tocinoic acid (0.5 mM) (Figure 2-1B) or somatostatin (0.2 mM) (Figure 2-1C) showed no obvious changes in their binding ability. Exposure to anti-PDI antibody up to 800 μg/ml also failed to influence adhesion (Figure 2-1D). However, treatment with 1.5 mM bacitracin resulted in a >80% inhibition of PMA induced adherence. These results are summarized in Table 2-1. Since the concentrations of DTNB, somatostatin, tocinoic acid and anti-PDI used were reported to block PDI activity (Couet J et al, 1996; Essex DW, 1995; Mandel R et al, 1993), these results suggested that bacitracin could inhibit α4β1 and α5β1 integrin-mediated cell adhesion and that this inhibition was not related to the inhibitory effects on conventional PDI.

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. As shown in Figure 2-2, 30 min later after bacitracin was washed away, the cells displayed a 40-45% recovery of adhesive potential in both PMA stimulated and basal groups. Using trypan blue staining of the cells showed no significant difference in the viability in both bacitracin treated and the control groups. These results exclude the

Figure. 2-1A

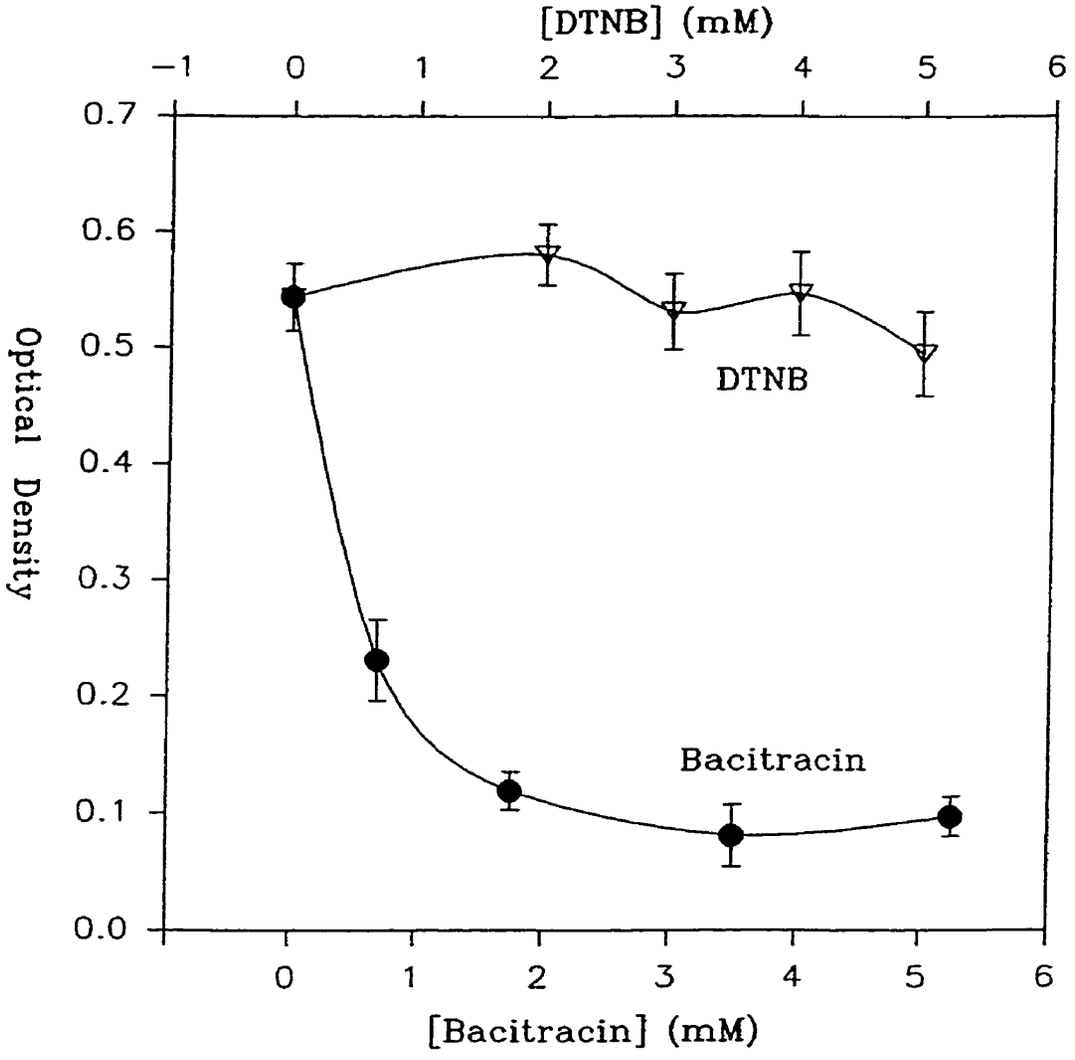

[DTNB] (mM)
−1
0
1
2
3
4
5
6
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density
DTNB
Bacitracin
0
1
2
3
4
5
6
[Bacitracin] (mM)

Figure 2-1A: The effects of bacitracin and DTNB on lymphocyte adherence to fibronectin. IL-2 dependent T cells were treated with indicated concentration of bacitracin or DTNB at 37^0C for 30 min and then, in the presence of bacitracin or DTNB, were stimulated with 50 ng/ml PMA at room temperature for 5 min. The cells were assayed for adherence to immobilized fibronectin. The data shown here are the mean of sextuplicate assays that have been repeated at least three times.

Figure 2-1B

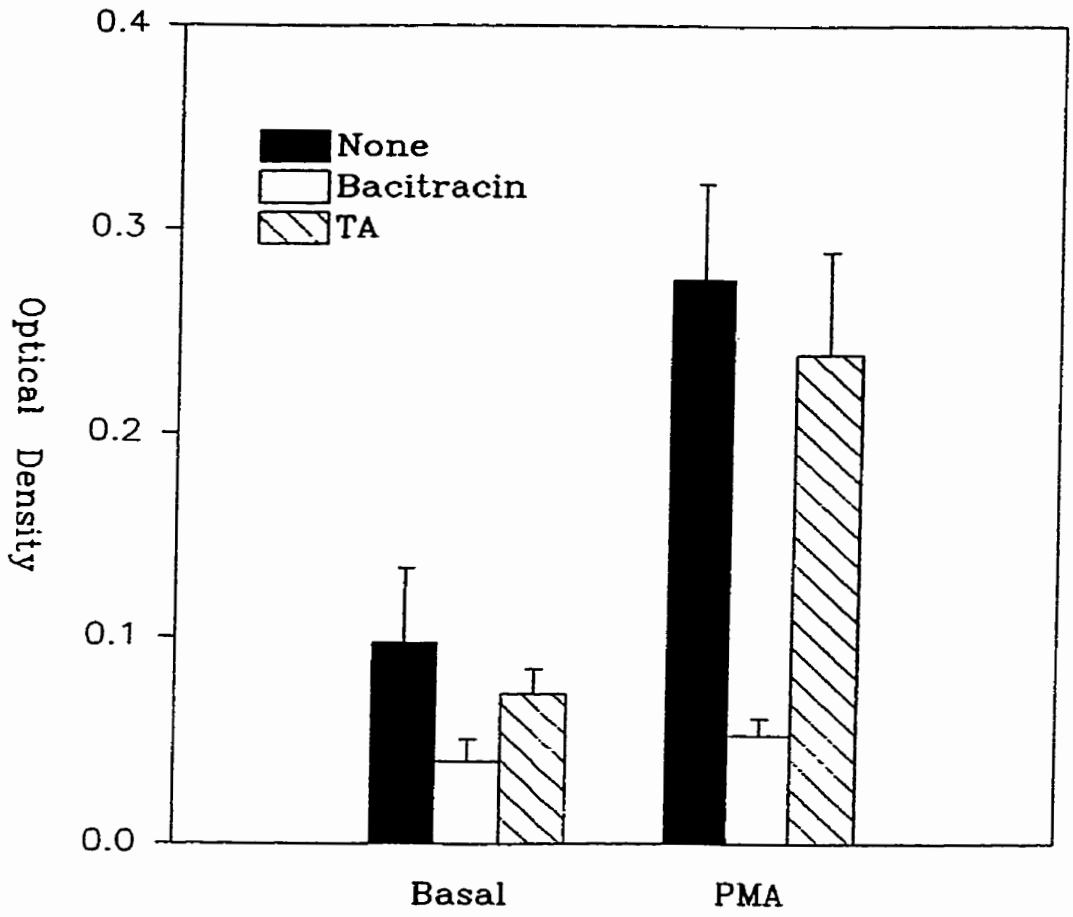


Figure 2-1B: The effects of tocinoic acid on lymphocyte adherence to fibronectin. Untreated or 0.5 mM tocinoic acid-treated or 3.5 mM bacitracin-treated IL-2 dependent T cells were, in the presence of tocinoic acid or bacitracin, either stimulated with 50 ng/ml PMA or unstimulated, and then assessed for binding to fibronectin. The results shown here are the mean and SE of sextuplicate assays that have been repeated at least three times.

Figure 2-1C

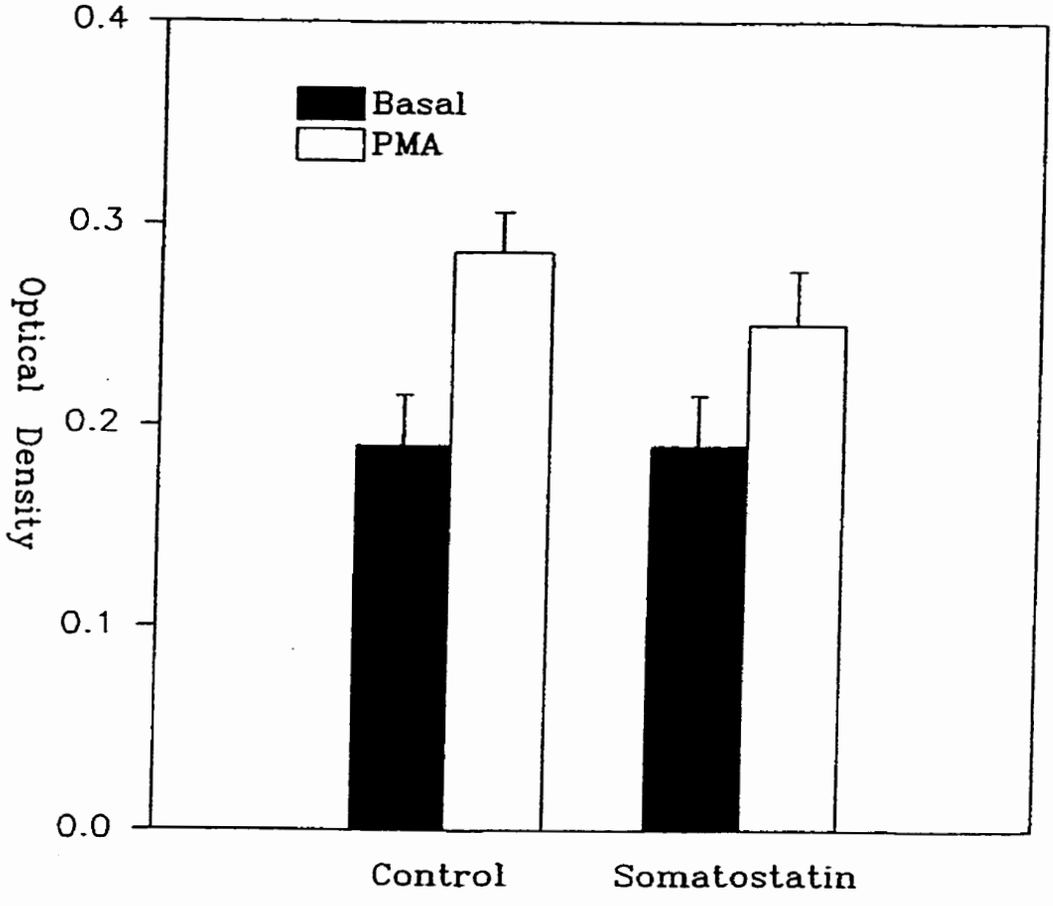

0.4
0.3
0.2
0.1
0.0
Optical Density
Basal
PMA
Control
Somatostatin

Figure 2-1C: The effects of somatostatin on lymphocyte adherence to fibronectin. Untreated or 0.2 mM somatostatin-treated or 3.5 mM bacitracin-treated IL-2 dependent T cells were, in the presence of somatostatin or bacitracin, stimulated with 50 ng/ml PMA or unstimulated and assayed for binding to fibronectin. The data shown here are the mean and SE of sextuplicate assays that have been repeated at least twice.

Figure. 2-1D

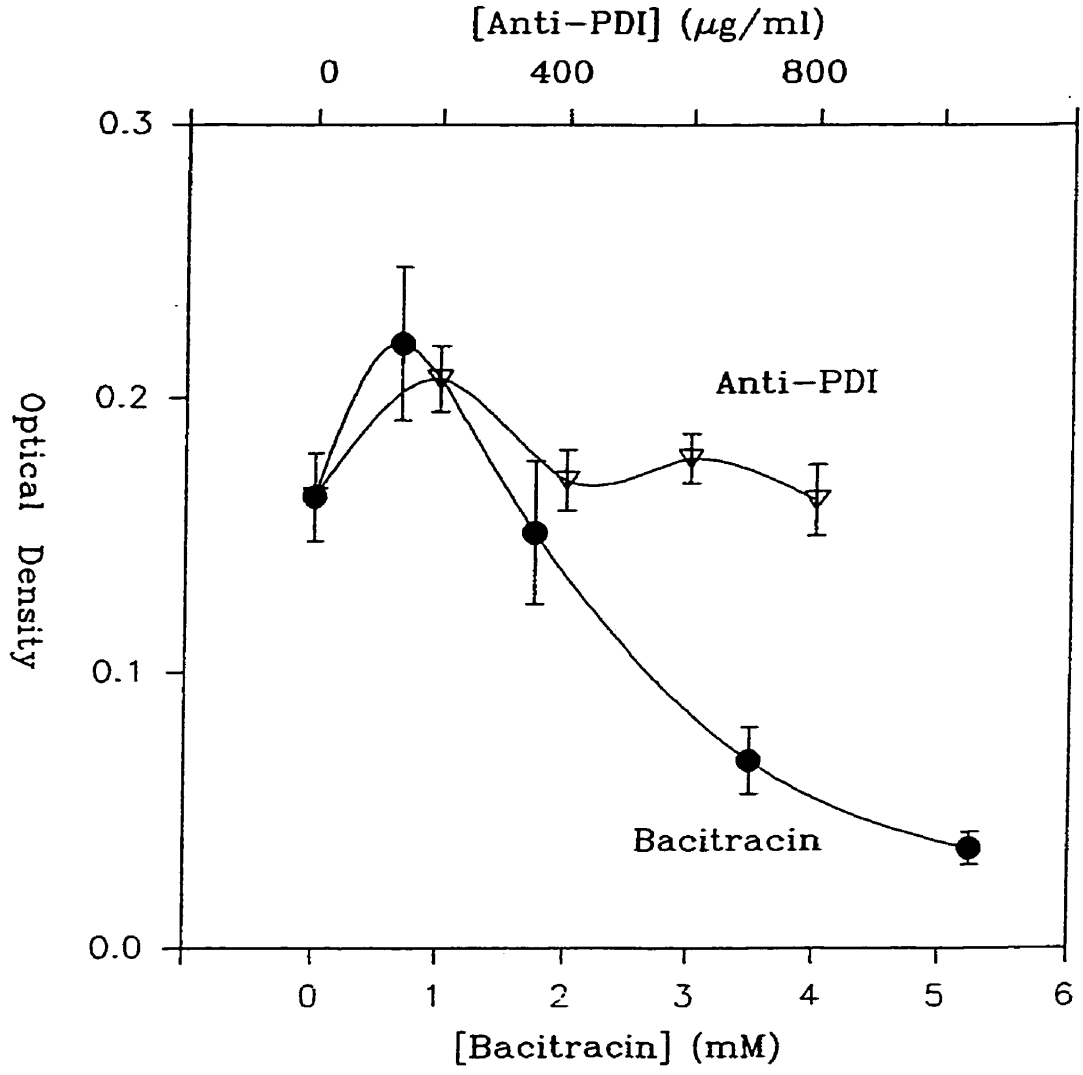

[Anti-PDI] (μg/ml)
0
400
800
0.3
0.2
0.1
0.0
Optical Density
Anti-PDI
Bacitracin
0
1
2
3
4
5
6
[Bacitracin] (mM)

Figure 2-1D: The effects of anti-PDI on lymphocyte adherence. IL-2 dependent T lymphocytes were treated with the indicated concentrations of anti-PDI (clone RL 77) or bacitracin at 37^{0}C for 30 min and then, in the presence of the indicated inhibitors, were assayed for adherence to fibronectin following PMA stimulation. The results for all experiments are the mean of sextuplicate assays that have been repeated at least two times.

Table 2-1. Effects of PDI inhibitors on $\alpha 5\beta 1$ integrin-mediated cell adhesion to fibronectin

Reagents	Concentration	Effect on cell adhesion
DTNB*	5 mM	-
Tocinoic Acid	0.5 mM	-
Somatostatin A	0.2 mM	-
Anti-PDI (RL 77)	800 μg/ml	-
PDI	100 μg/ml	-
Bacitracin	3.5 mM	+

*DTNB: dithiobisnitrobenzoic acid

"-": has no obvious effect

"+": has obvious effect

Figure 2–2

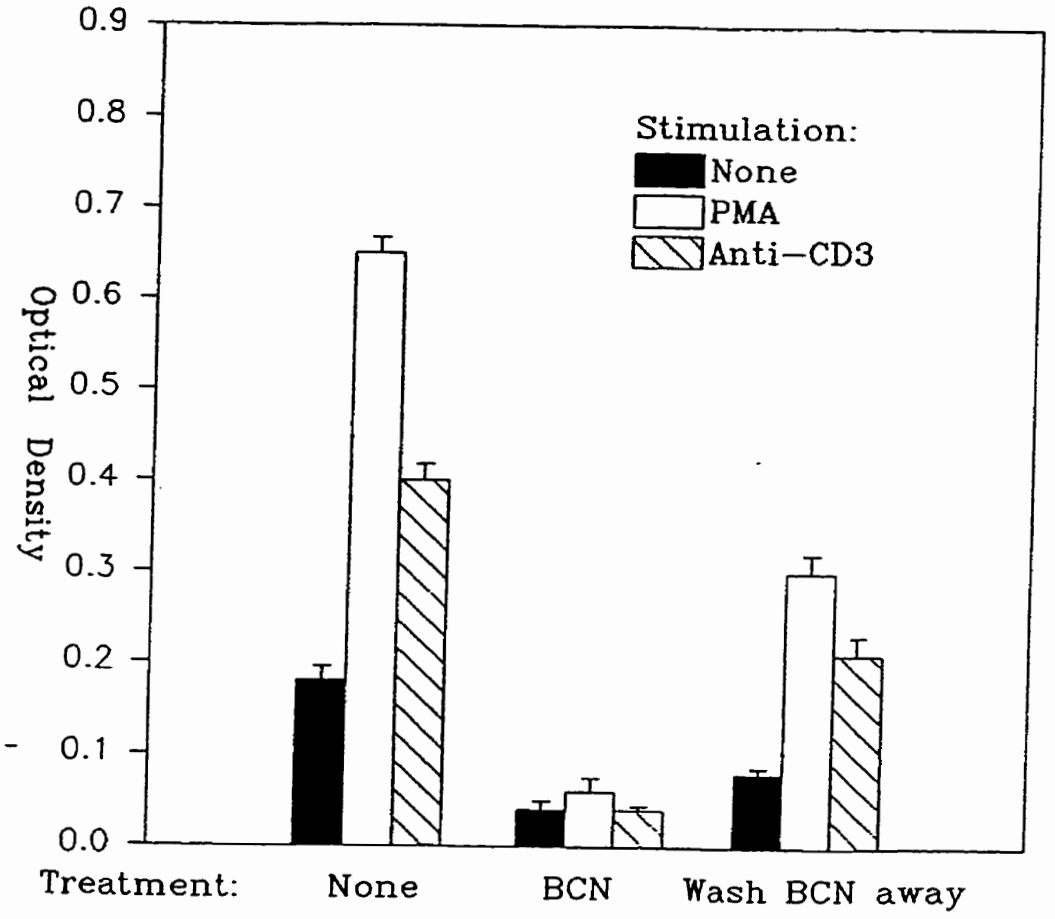

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density
Stimulation:
None
PMA
Anti–CD3
Treatment:
None
BCN
Wash BCN away

Figure 2-2. The reversibility of bacitracin effects on cell adhesion. After treated with 3.5 mM bacitracin at 37^0C for 30 min, IL-2 dependent T cells were washed twice with fresh media to remove the bacitracin, and were then incubated at 37^0C for 30 min in fresh media. Cells were assayed for spontaneous, anti-CD3 monoclonal antibody-induced and PMA-induced adherence to fibronectin. The results shown here are the mean and SE of sextuplicate assays that have been repeated at least two times.

possibility that bacitracin induced inhibition to α5β1 was related to toxic effect on the cells.

2. The spectrum and specificity of bacitracin induced inhibition of integrin functions:

Bacitracin inhibited α4β1 and α5β1 integrin mediated cell adhesion to fibronectin. In order to further define the possible mechanisms of such inhibitory effects, experiments on the spectrum and specificity of bacitracin-induced inhibition of integrin functions were performed.

2.1 Bacitracin blocks cell adhesion mediated by β1 integrins:

It has been demonstrated that the β1 subunit of integrins can combine with different α subunits to form different integrins. These different integrins bind to different ligands (Hynes RO, 1992). In order to determine whether bacitracin could inhibit these different combinations of α subunit with β1 subunit of integrin-mediated cell adhesion, the effects of bacitracin on different integrins on human lymphocytes were analyzed. As shown in the Table 2-2, α4β1, α5β1 and α6β1 integrin-mediated cell adhesions were very sensitive to bacitracin. Almost 90% cell adhesion was inhibited at less than 1 mM concentration of bacitracin. The IC50 (concentration required to induce 50% inhibition of cell adhesion to immobilized ligand) required was about 0.5 mM. However, α2β1 integrin was the least sensitive among all β1 integrin-mediated cell adhesion tested. Its IC50 was about 5-6 mM, almost 10 times higher than those for α4β1, α5β1 and α6β1.

Table 2-2: The effects of bacitracin on integrin–dependent binding to selected ligands

Cells	Integrin	Ligand	IC50*
PBL	α4/α5β1	Fibronectin	500-1000 μM
PBL	α2β1	Collagen	5-6 mM
PBL	α4β1	VCAM-1	500-1000 μM
Jurkat	α4β1	Fibronectin 40 kD (CS-1)	500-1000 μM
Jurkat	α5β1	Fibronectin 120 kD (CBD)	500-1000 μM
PBL	α6β1	Laminin	500-1000 μM

*IC50: Concentration required to induce 50% inhibition of cell adhesion to immobilized ligand.

2.2 Specificity of bacitracin effects

Bacitracin inhibited all β1 integrin-mediated cell adhesion tested to date. This raised the question of whether bacitracin induced inhibitory effects were integrin β chain specific? Understanding this question is important for further understanding the possible mechanisms of bacitracin induced inhibitory effect on integrin. In order to answer this question, human B cell line JY and 8866 cells expressing αLβ2, α4β7 and αvβ3 integrins but little or no β1 subunit (Stupack DG et al, 1991; Stupack DG et al, 1992) were examined for their sensitivities to bacitracin.

JY cells were pretreated with bacitracin for 30 min, stimulated by PMA and assayed for their ability to bind recombinant human ICAM-1 coated 96-well plates. Binding of JY cells to ICAM-1 under these conditions is mediated by β2 integrins as the binding could be inhibited by anti-β2 integrin monoclonal antibodies (data not shown). Pretreatment of cells with bacitracin inhibited cell adhesion by more than 80% at 3.5 mM concentration. The IC50 was about 500-1000 μM (Figure 2-3), suggesting that β2 integrin-mediated cell adhesion to ICAM-1 could also be inhibited by bacitracin.

8866 cells bind to 40 kD fibronectin through α4β7 integrin while they employ αvβ3 integrin to 120 kD fibronectin fragment (Stupack D. et al, 1992; Stupack D. et al, 1991). When treated with bacitracin, α4β7 integrin-mediated adhesion was greatly inhibited. The IC50 was around 500-1000 μM. However, αvβ3 integrin-mediated cell adhesion was increased at low concentrations of bacitracin and reached the plateau at 1 mM and then gradually reduced. The IC50 for αvβ3 dependent adhesion was more than 8 mM (Figure 2-4). Since these two experiments (i.e. α4β7 and αvβ3 integrin-mediated

Figure 2–3

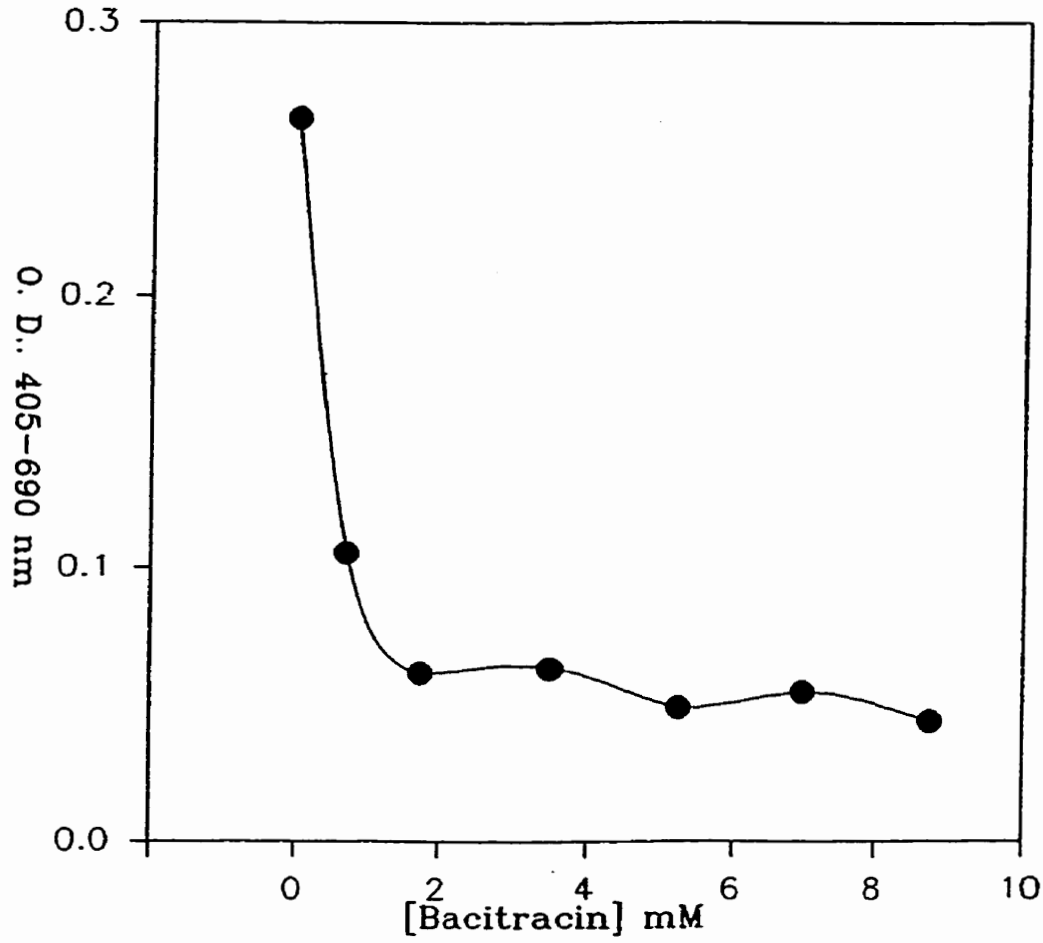


Figure 2-3. The effects of bacitracin on β2 integrin-mediated cell adhesion to ICAM-1. RPMI 8866 cells were pretreated with the indicated concentrations of bacitracin for 30 min at 37^0C and assayed for adherence to immobilized ICAM-1. The data shown here are the mean of sextuplicate assays that have been repeated at least two times.

Figure 2–4

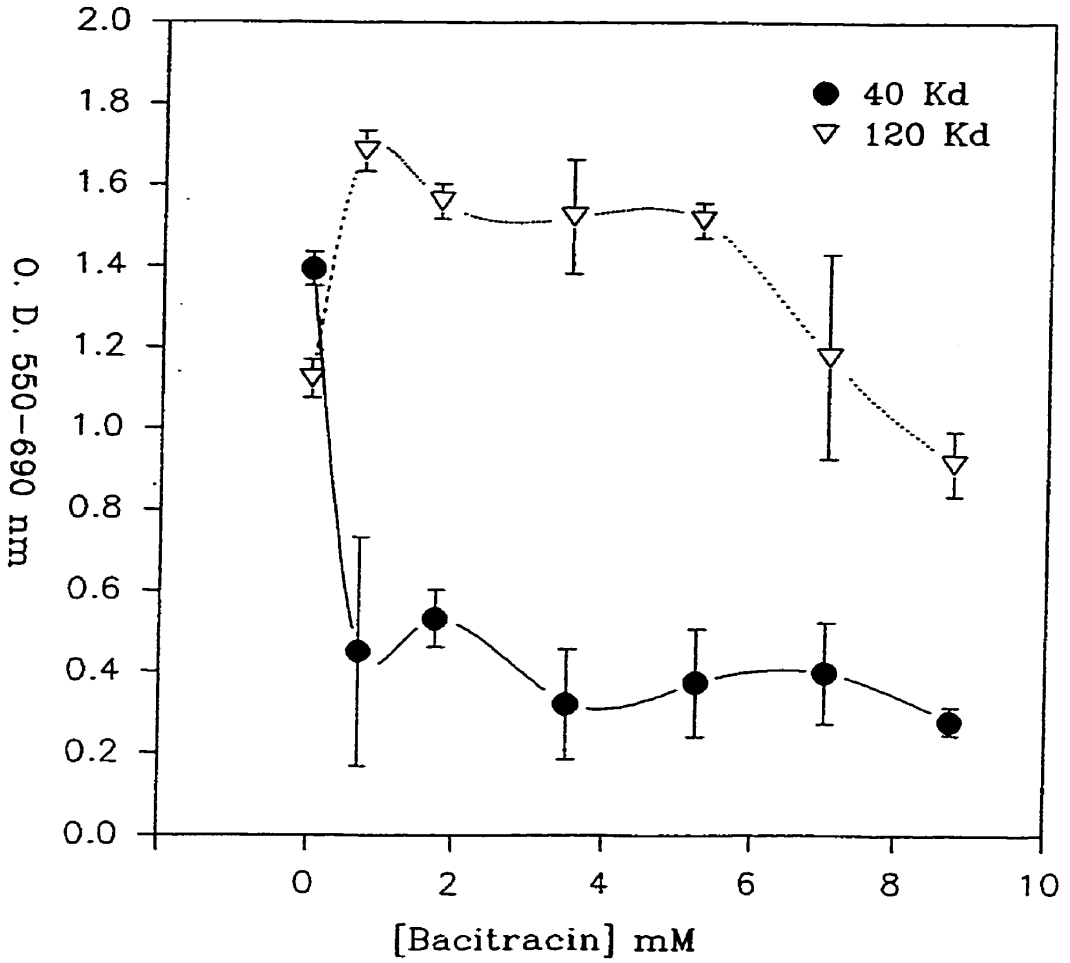

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O. D. 550–690 nm
40 Kd
120 Kd
0
2
4
6
8
10
[Bacitracin] mM

Figure 2-4. The effect of bacitracin on RPMI 8866 cells adherence to 40 kD and 120 kD fibronectin fragment. 50 ng/ml PMA-stimulated RPMI 8866 cells were pretreated with the indicated concentrations of bacitracin and assessed for their binding to 40 kD and 120 kD fibronectin fragment. The results for all experiments are the mean of sextuplicate assays that have been repeated at least two times.

binding assays) were performed under exactly the same conditions, this minimized the possible experimental variation. This experiment further indicated that the inhibitory effect of bacitracin to integrin mediated cell adhesion was not due to the interference of cell viability. It also indicated that bacitracin could selectively inhibit $\alpha 4\beta 7$ but less extent to $\alpha v\beta 3$ integrin-mediated cell adhesion.

Therefore, bacitracin shows potent selective inhibitory effects on $\beta 1$ and $\beta 7$ but to less extent to $\beta 3$ integrin-mediated cell adhesion. This inhibitory effect seems not to be integrin α subunit specific since bacitracin can inhibit αL, $\alpha 4$, $\alpha 5$ and $\alpha 6$ integrin-mediated cell adhesion. The detailed mechanisms of this inhibition require further exploration.

3. The mechanisms of bacitracin induced inhibition of $\beta 1$ integrin-mediated cell adhesion:

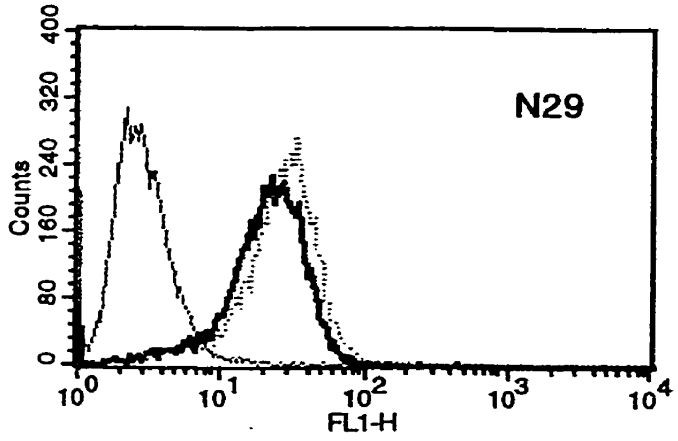
As mentioned in the introduction, integrins on the cell surface can be regulated quantitatively and qualitatively. To test the possible mechanisms of bacitracin-mediated inhibitory effects on $\beta 1$ integrin-mediated cell adhesion, the following experiments were performed.

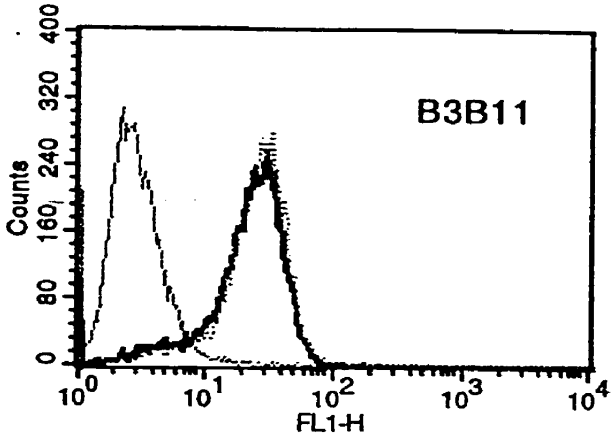
3.1 Bacitracin effects on integrin expression level on cell surface

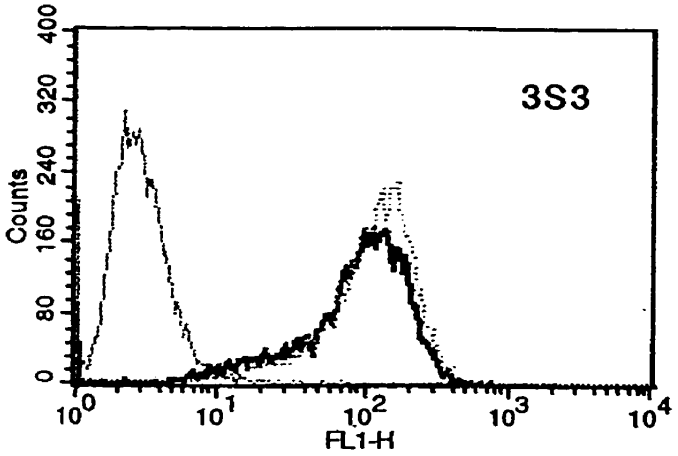
We chose $\beta 1$ integrin as our major target because $\beta 1$ integrin has been extensively investigated in our laboratory.

The effects of bacitracin on integrin expression levels on cell surface were determined by comparing the staining patterns of bacitracin-treated cells with those of control cells (Ni HY et al, 1998a). Therefore, anti-β1 integrin monoclonal antibodies, which recognize different regions of β1 integrins were used for the FACS analysis. Monoclonal antibodies N29 and JB1A recognize epitopes located in the N-terminus of the β1 integrin (residues 15-54 and 82-87 respectively) (Ni HY et al., 1998a; Ni HY et al., 1998b). Monoclonal antibody B3B11 recognizes an epitope located proximal to the transmembrane region of β1 integrin extracellular domain (residues 666-668) (Wilkins JA et al., 1995). Monoclonal antibody B44 recognizes residues from 355-425 (unpublished data) while monoclonal antibody 3S3 recognizes a conformational epitope (Gao JX et al., 1995). Following the treatment with 3.5 mM bacitracin, the expression level of the epitopes recognized by N29 and JB1A (N-terminal epitope), B3B11 (C-terminal epitope near transmembrane) and 3S3 (conformational epitope) were unchanged (Figure 2-5). This result suggested that bacitracin might not change β1 integrin expression level. However, the expression level of B44 epitope was increased significantly, indicating that there might be local conformational changes in β1 integrins.

Monoclonal antibody B44 has been shown to recognize the so called cation-ligand-induced-binding-site (CLIBS) epitope in β1 integrin (Ni HY, manuscript), i. e. integrin ligands or divalent cations can induce the conformational changes of integrin, which expose B44 epitope. Since bacitracin could induce expression of B44 epitope, this raised a question whether bacitracin could induce β1 integrin conformational changes similar to those caused by β1 integrin ligands or divalent cations. Understanding this


N29
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H


B3B11
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H


3S3
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

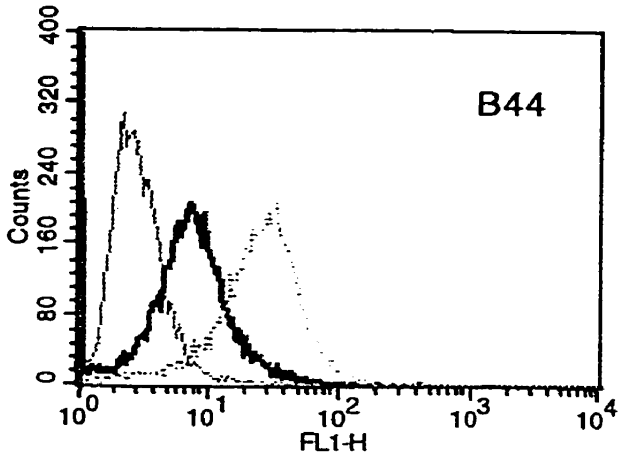

B44
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 2-5. The effect of bacitracin on β1 integrin expression levels and conformation. Untreated (solid dark line) or 3.5 mM bacitracin-pretreated (dashed line) cells were incubated with the indicated antibodies, and were examined by flow cytometry for β1 integrin expression level. Note the staining patterns of antibody N29, B3B11 and 3S3 in the presence of bacitracin are totally superimposed on their corresponding untreated counterparts. However, in the presence of bacitracin, B44 epitope expression is increased. The light line in each histogram represents the negative control.

question may help us to further explore the mechanisms of bacitracin induced inhibitory effects on integrins. In order to test this possibility, monoclonal antibodies recognizing other CLIBS on β1 integrin were tested.

3.2 Bacitracin causes β1 integrin conformational change, leading to increased expression of CLIBS on β1 integrin:

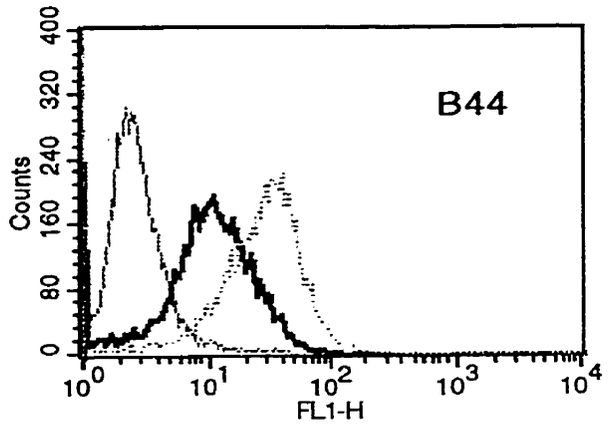
CLIBS on β1 integrin have been reported by several groups of researchers (Bazzoni G et al, 1995; Yednock TA et al, 1995, Luque A et al, 1996; Mould AP et al, 1995b, Wilkins JA et al, 1995). A panel of monoclonal antibodies that were thought to recognize CLIBS epitopes on β1 integrin had been mapped for their binding sites on the β1 chain. One of these binding sites was located at 354-425 on β1 integrin. These antibodies included mAb B44, HUTS4, HUTS21, 15/7 and 13B9 (Willian HJ et al., 1994; Faull RJ, 1995). However, monoclonal antibody B44 recognizes a linear epitope while HUTS21, 15/7 and 13B9 recognize a conformational epitope that are dependent on this region of the β1 molecule. The antibodies 15/7, HUTS21 and 13B9 caused a 40-64% reduction of mAb B44 binding to its epitope. In HUTS21, 15/7 and 13B9 pre-treated Jurkat cells mAb B44 binding is fully inhibited (Ni HY unpublished data). All these data suggest that the epitopes recognized by these different antibodies are in close spatial proximity but do not overlap with each other. Divalent cations such as Mn^{++}, Mg^{++} and β1 integrin ligands such as the RGD peptide can also induce the increased expression of CLIBS on β1 integrin (Ni HY et al, 1998a; Willian HJ et al 1994; Faull RJ, 1995).

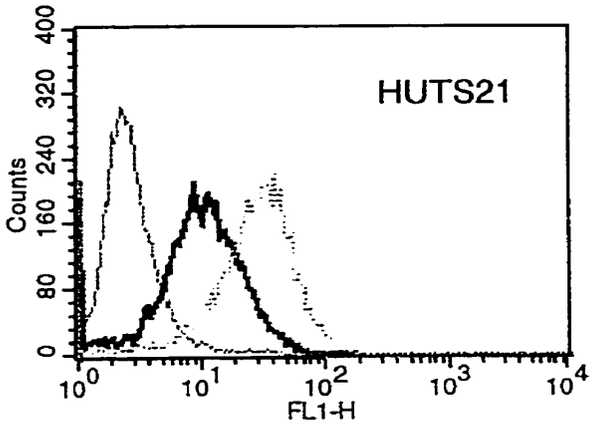
Treatment of Jurkat cells with bacitracin resulted in an increased expression of the epitopes recognized by HUTS4, HUT21 and 15/7. The expression level of HUTS4, HUTS21 and 15/7 were almost the same as B44 epitope (Figure 2-6).

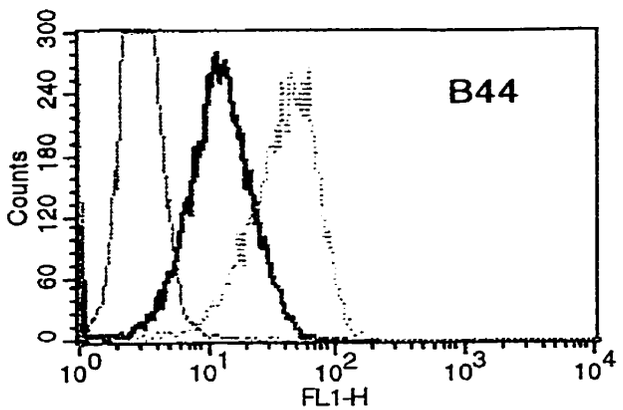
Thus, we concluded that bacitracin could cause similar β1 integrin conformational changes as divalent cation or β1 integrin ligands.

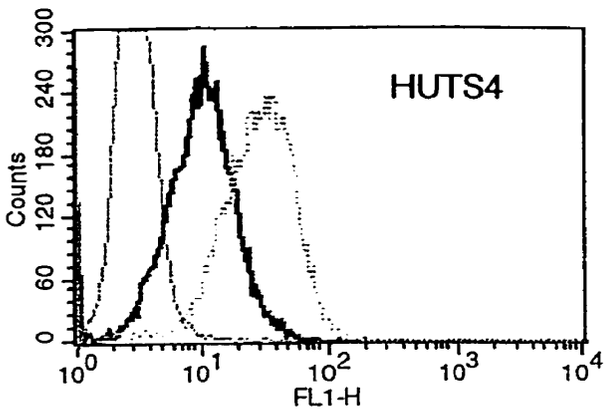
3.3 The possible role of PDI in inducing CLIBS expression on β1 integrin:

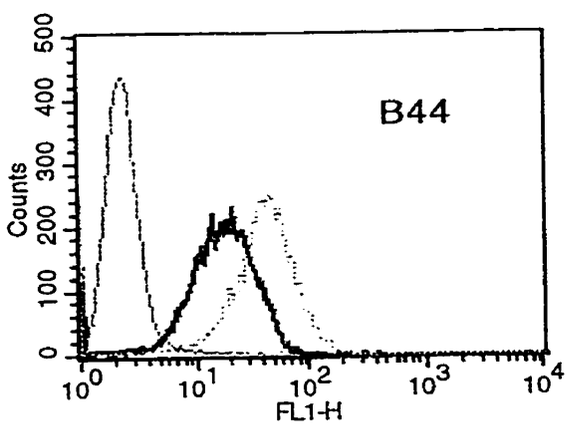
β1 integrin is presumed to contain 28 intrachain disulfide bridges which are probably important for maintaining the β1 integrin conformational structure (Hemler ME 1990). Since PDI is thought to be a critical component in protein disulfide bridge regulation (Luz JM, 1996), one possibility is that bacitracin induced B44 epitope is mediated by the inhibitory effect of bacitracin on PDI function. To test this possibility, Jurkat cells were pre-treated with PDI inhibitor tocinoic acid, somatostatin or inhibitory anti-PDI monoclonal antibody (RL 77) and were tested for β1 integrin epitope expressions. In the presence of bacitracin or tocinoic acid, B44 epitope expression was increased, N29 epitope was also increased by tocinoic acid (Figure 2-7) and somatostain (data not shown) treated group although not as much as B44 epitope, while B3B11, JB1A and 3S3 epitopes were unchanged. These results were completely different from those in the presence of inhibitory mAb to PDI in which both 3S3 and JB1A decreased a little bit, B44 and N29 epitopes were unchanged (Figure 2-8). These results suggest that the changes in B44 epitope expression levels are not related to the inhibitory effects on conventional PDI.


B44
Counts
FL1-H


HUTS21
Counts
FL1-H


B44
Counts
FL1-H


HUTS4
Counts
FL1-H


B44
Counts
FL1-H

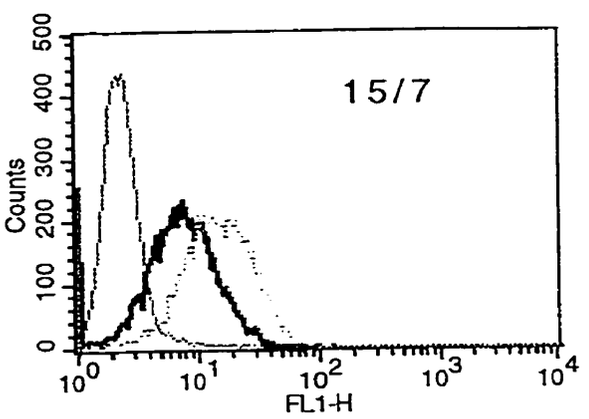
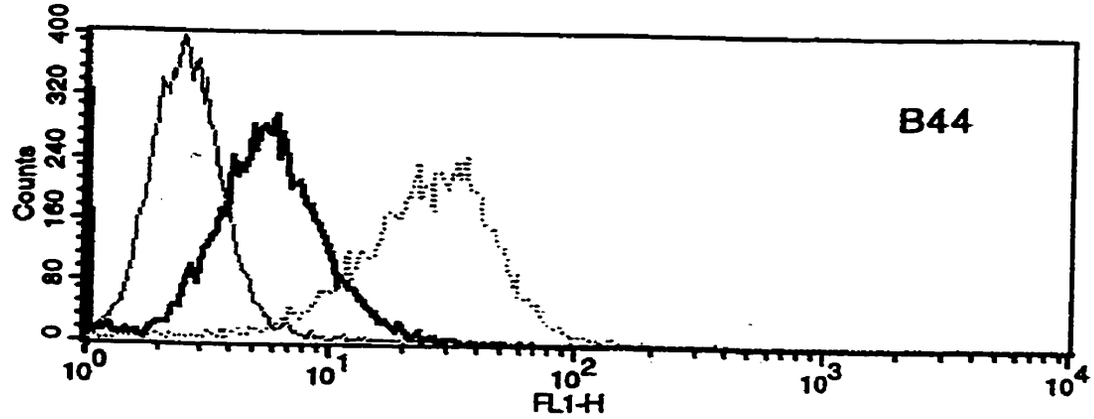
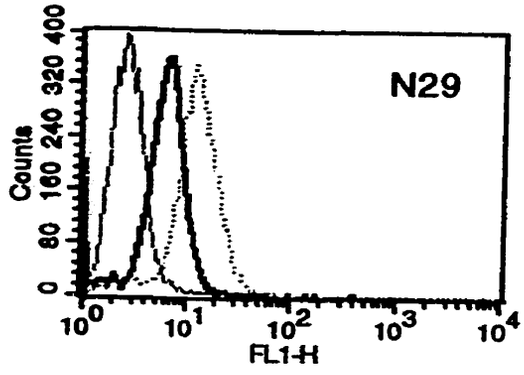
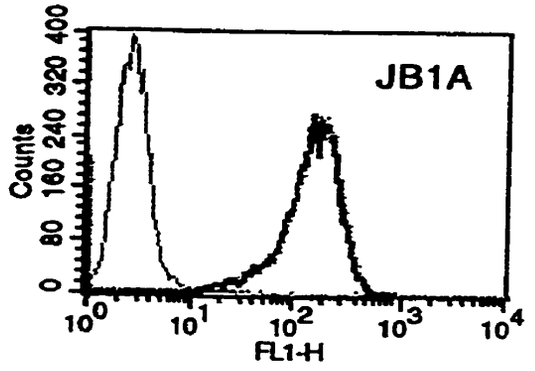
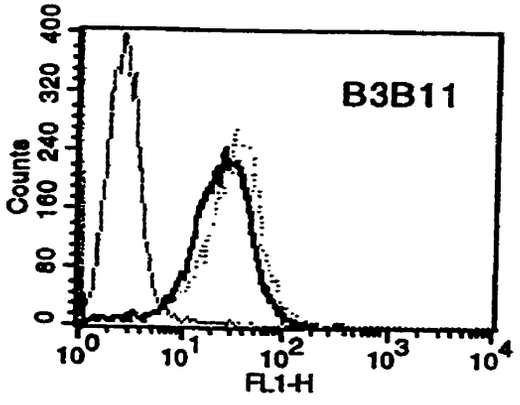

15/7
Counts
FL1-H

Figure 2-6. The effect of bacitracin on epitope CLIBS expression on β1 integrin. Cells were incubated with indicated monoclonal antibody to β1 integrin in the absence (solid dark line) or presence (dashed line) of 3.5 mM bacitracin and examined by flow cytometry. The light line in the histogram represents the negative control.


B44
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H


N29
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H


JB1A
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H


B3B11
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

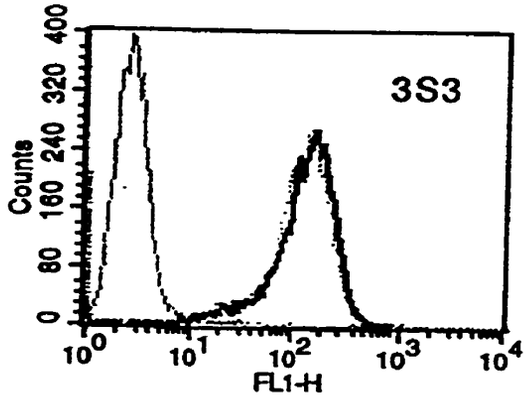

3S3
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 2-7: The effect of tocinoic acid on epitope expression on β1 integrin. Jurkat cells were incubated with indicated monoclonal antibody to β1 integrin in the presence (dashed line) or absence (solid dark line) of 0.5 mM tocinoic acid. The epitope expression level was examined by flow cytometry. The light line in the histogram represents the negative control.

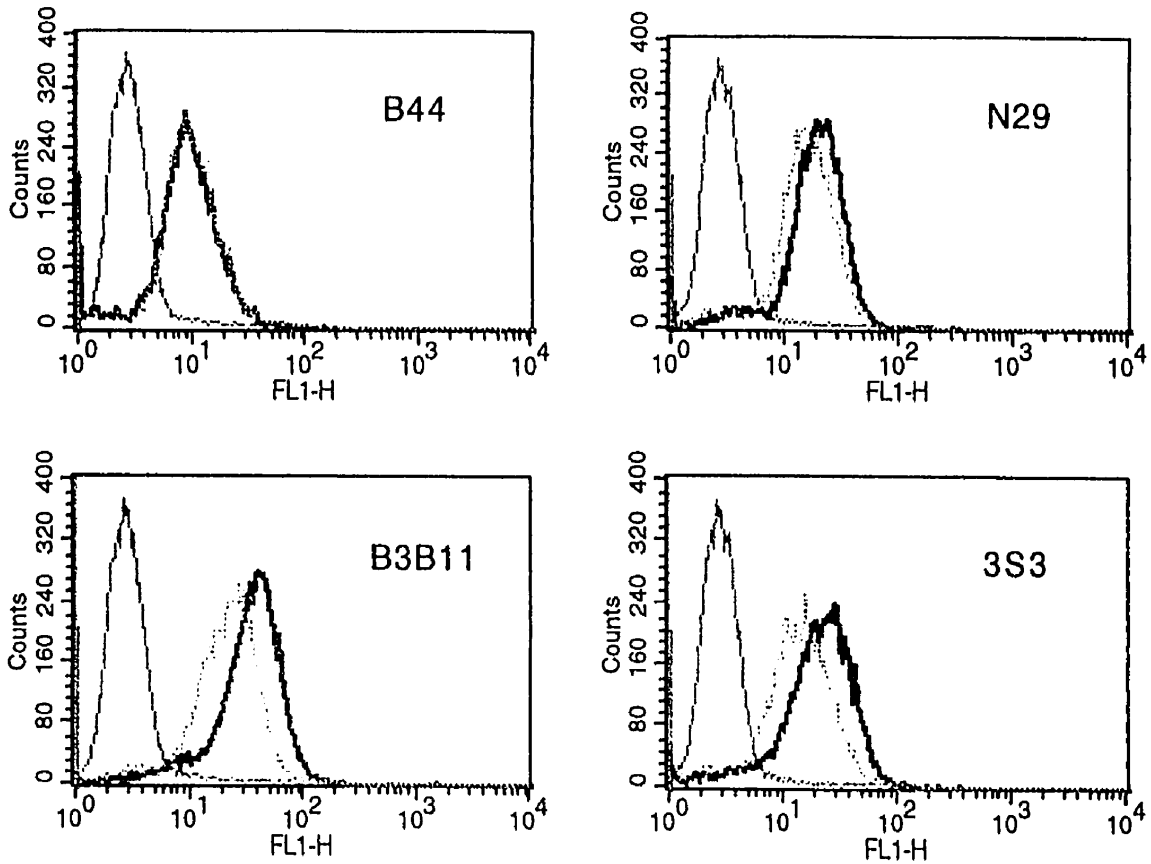

B44
N29
B3B11
3S3
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 2-8: The effect of inhibitory mAb against PDI (RL77) on epitope expression on β1 integrin. Jurkat cells were incubated with monoclonal antibody to β1 integrin, B44, N29, B3B11 and 3S3 in the presence or absence of 800 μg/ml anti-PDI mAb. The epitope expression level was examined by flow cytometry. The light line in the histogram represents the negative control. The basal expression is marked with the solid dark line, the epitope expression in the presence of mAb against PDI is indicated by the dashed line.

Taken together, bacitracin does not change integrin expression level on cell surface. Instead, it causes integrin conformational changes and results in increased expressions of CLIBS, which are similar to divalent cation and integrin ligand-induced β1 integrin conformational changes. These conformational changes are not mediated by conventional PDI. These results suggest that bacitracin may regulate integrin functional states on the cells.

4. The possible pathway(s) of bacitracin induced inhibitory effects on β1 integrins:

Integrin functional regulation on the cell surface can be achieved by "Inside-out" or "Outside-in" pathways. To further elucidate the mechanisms of bacitracin-induced inhibitory effects on β1 integrin, the possible pathways used by bacitracin were tested.

4.1 Bacitracin directly inhibits the interaction between integrin and integrin ligand

Since both PMA stimulated T cell adherence and the adherence of the non-PMA-treated cells were inhibited by pretreatment with 3.5 mM bacitracin shown in Figure 2-2, this implies that the inhibition caused by bacitracin may possibly occur at integrin-ligand interaction or post-activation event. To test this possibility, cells were treated with stimulatory mAb against β1 integrin, B44, or Mn++ in the presence of bacitracin and assayed for adherence to fibronectin. The adhesion induced by mAb B44 and Mn^{++} (Figure 2-9) was fully inhibited by bacitracin. The induction of adherence by anti-β1 mAb TS2/16 was also inhibited by bacitracin (date not shown). These results suggested that the site of action was located in integrin-ligand interaction or post-activational β1 step.

Figure 2-9

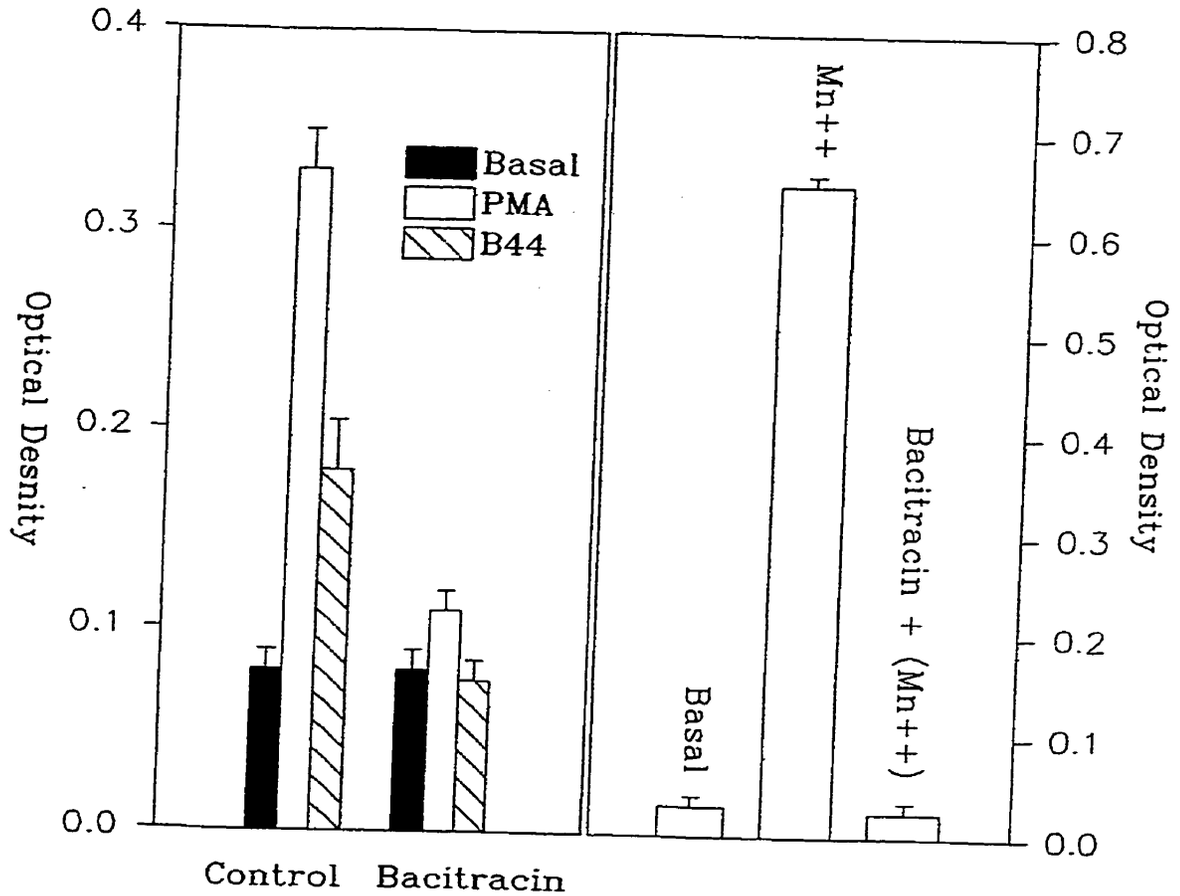

0.4
0.3
0.2
0.1
0.0
Optical Desnity
Basal
PMA
B44
Control
Bacitracin
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density
Mn++
Basal
Bacitracin + (Mn++)

Figure 2-9: The effects of bacitracin on activated cell adhesion. 10 μg/ml stimulatory mAb B44 or 100 μg/ml Mn^{++} activated cells were treated with 3.5 mM bacitracin or untreated and assayed for binding to fibronectin.

In order to further determine the site of bacitracin action, the ability of cells to bind soluble fibronectin was assessed as a direct test of integrin competence. Treatment of K562 cells with bacitracin resulted in a complete inhibition of soluble fibronectin binding, as shown in FACS results, implying that the direct interaction between integrin and ligand was the site of action rather than post-receptor occupancy events required for cell adherence (Figure 2-10).

As a direct test of the above prediction, the effect of bacitracin on the binding of fibronectin to purified β1 integrin was examined. At 3.5 mM bacitracin, more than 80% of fibronectin binding was inhibited while the background binding of fibronectin was relatively unaffected (Figure 2-11). This result suggests that bacitracin can directly interfere with fibronectin binding to purified β1 integrin.

4.2 Comparing CLIBS expression in βI integrin induced by bacitracin and integrin ligand:

The above results raise a question whether bacitracin can mimic integrin ligand binding to the integrin?

To answer this question, Jurkat cells were treated with RGD peptide ligand or bacitracin in the presence or absence of the divalent cation chelate, EDTA. As expected, RGD peptide ligand could induce the expression of B44 epitope, this ability was greatly reduced in the presence of EDTA (Figure 2-12). This was consistent with the finding that

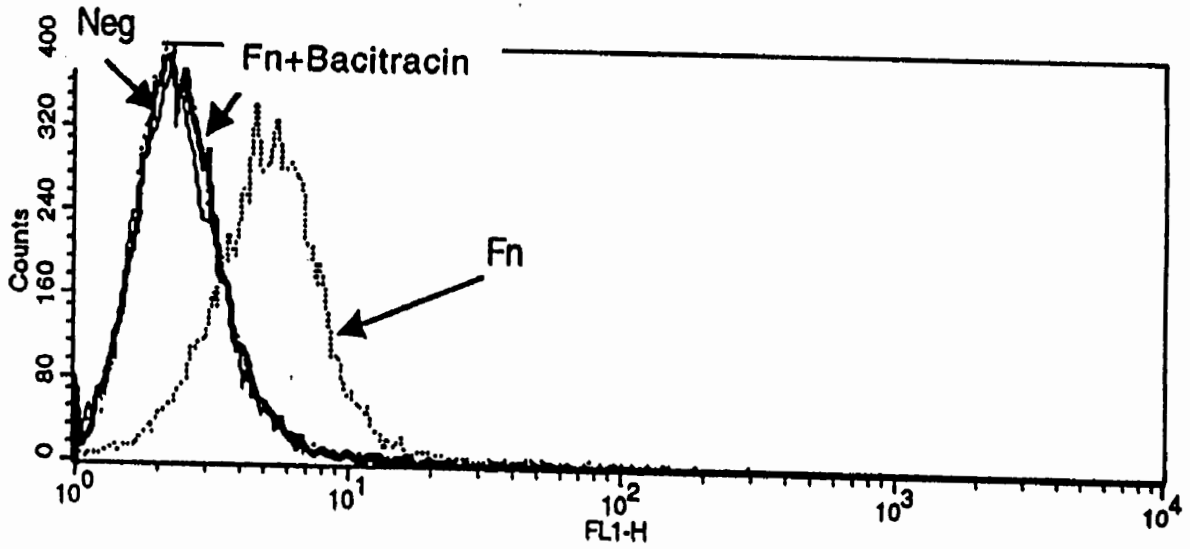

Neg
Fn+Bacitracin
Fn
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 2-10: The effect of bacitracin on the binding of soluble fibronectin to cell surface $\alpha 5\beta 1$ integrin. K562 cells were incubated with biotinylated soluble fibronectin in the presence (solid dark line) or absence (dashed line) of 3.5 mM bacitracin for 30 min. The cells were then washed and were reacted with FITC-labeled avidin. The binding of soluble fibronectin was examined by FACS. The negative control binding is indicated by the solid light line.

Figure 2-11

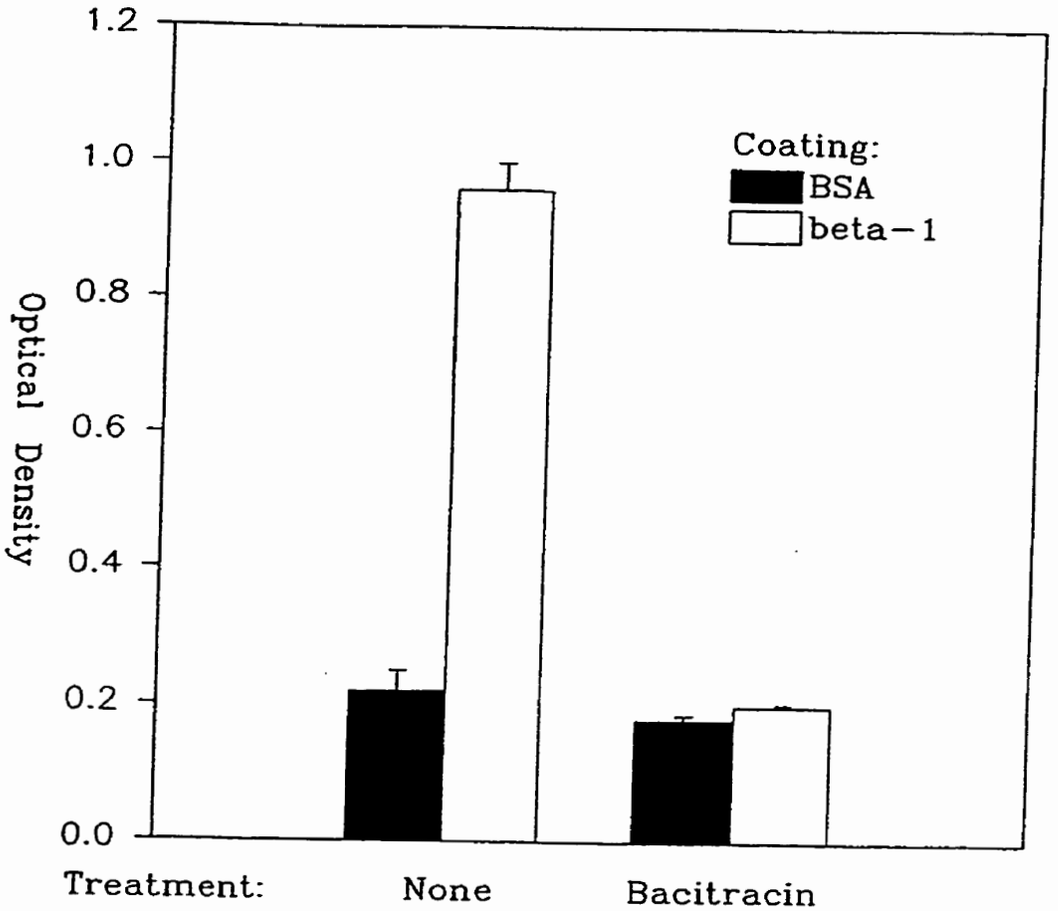

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density
Coating:
BSA
beta-1
Treatment:
None
Bacitracin

Figure 2-11: The effect of bacitracin on ligand binding to purified β1 integrin. Biotinylated fibronectin in the absence or presence of 3.5 mM bacitracin was assayed for binding to BSA or purified β1 integrin coated in microtiter wells. The O. D was determined at 405 nm following reaction with alkaline phosphatase conjugated avidin and substrate. The experiment was performed twice in replicates of five each time. The data shown here are the mean and the SE of one such experiment.

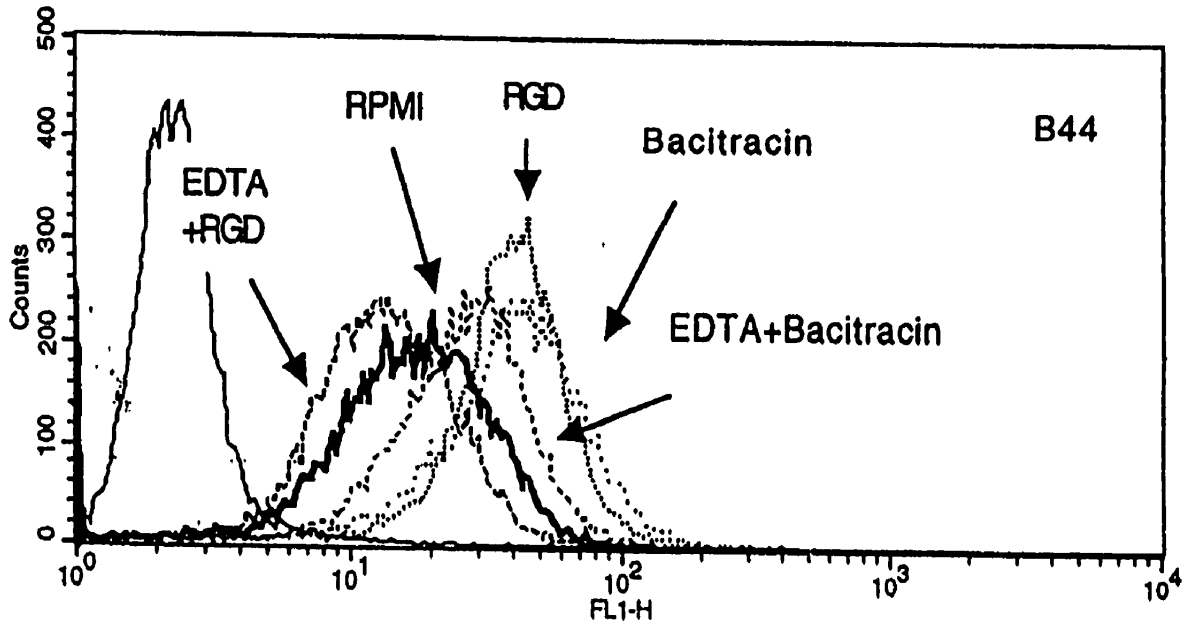

Counts
500
400
300
200
100
0
10^0
10^1
10^2
10^3
10^4
FL1-H
EDTA
+RGD
RPMI
RGD
Bacitracin
EDTA+Bacitracin
B44

Figure 2-12: The effect of divalent cation on bacitracin induced B44 epitope expression. Jurkat cells were incubated with bacitracin (3.5 mM) or 10 μM RGD peptide in the presence or absence of EDTA at 37^{0}C for 30 min and then were incubated with monoclonal antibody B44. B44 epitope expression levels were examined by flow cytometry. The basal expression level of B44 epitope was indicated as RPMI (solid dark line).

the interaction between β1 integrin and its ligands required divalent cation (Ni HY et al, 1998a). However, under the same conditions, bacitracin induced B44 epitope expression was marginally influenced. This result suggested that binding of bacitracin to β1 integrin is divalent cation independent, which is different from those of β1 integrin ligands.

Collectively, these results suggest that bacitracin may directly interact with integrin in a divalent cation independent manner. Since binding of cations to cation-binding sequences in integrin is thought to directly influence the binding of integrins to their ligands either by directly contributing a ligand-binding pocket or by creating a ligand-binding tertiary structure of the integrin molecule (Horton MA, 1996), it can be inferred that the interaction sites in integrin for bacitracin and integrin ligand are different. This suggests that bacitracin does not compete with integrin ligand to interact with integrins, but may cause integrin allosteric changes, leading to low affinity to integrin ligand.

5. Interactions between integrin and bacitracin:

To further elucidate whether bacitracin inhibited integrin function by direct competition with ligand or in an allosteric fashion, purified α5β1 integrin from placenta was coated on 96-well plate. Different concentrations of soluble biotin-labeled fibronectin were tested in the presence of different concentrations of bacitracin for its binding ability. As shown in Figure 2-13, in the absence of bacitracin, binding of soluble

Figure 2–13

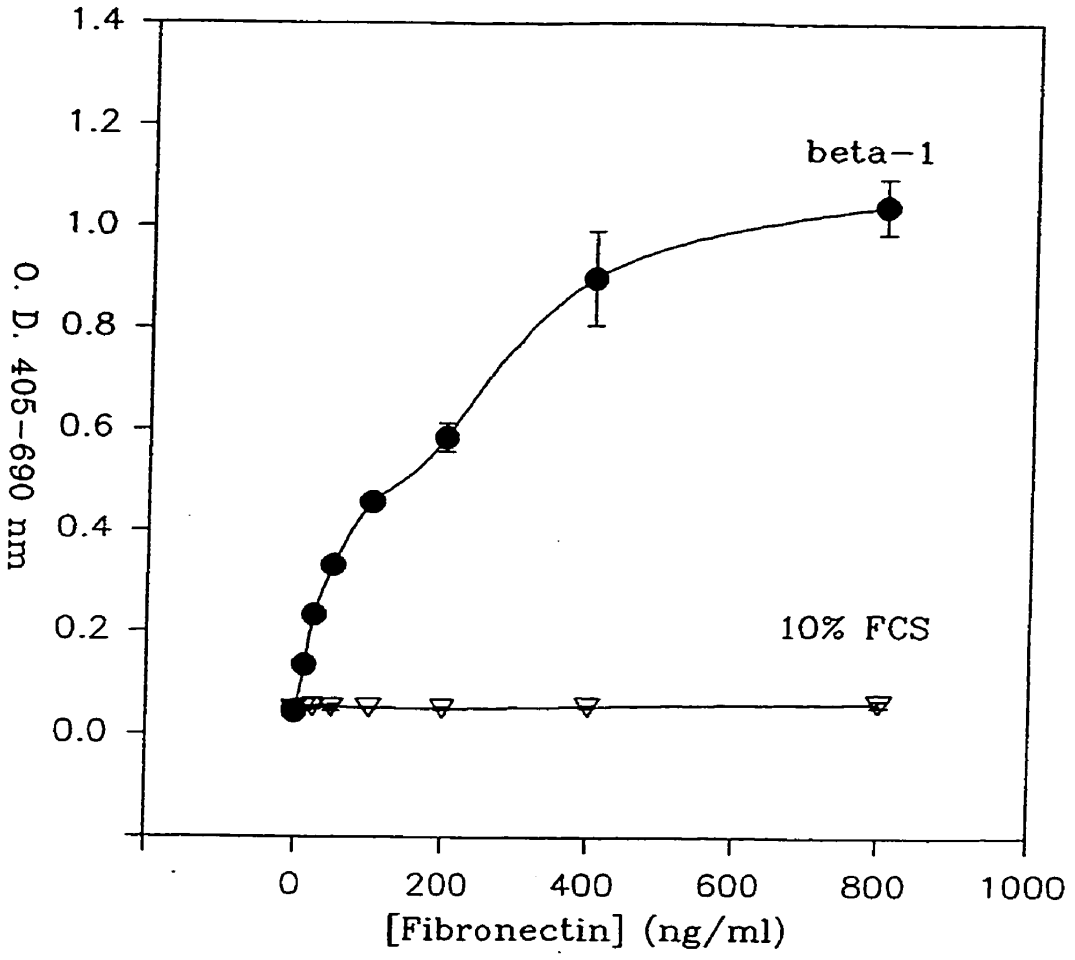

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O. D. 405–690 nm
beta–1
10% FCS
0
200
400
600
800
1000
[Fibronectin] (ng/ml)

Figure 2-13: Soluble fibronectin binding to immobilized purified α5β1 integrins. 100 ng of purified α5β1 integrin was coated in 96-well plate. The plate was then blocked by 10% heat-denatured FCS. Soluble fibronectin labeled with biotin was reacted with immobilized integrin at 30^0C for 3 hours. The level of bound fibronectin was then determined following reaction with alkaline phosphatase-conjugated avidin and substrate.

fibronectin was fibronectin-dose dependent. The binding plateau was reached at about 600 ng/ml of fibronectin and this binding could be inhibited by 100- fold excess of unlabeled fibronectin (data not shown), reflecting that this binding was fibronectin specific. In the presence of 100 μg/ml bacitracin, the binding ability of fibronectin was strongly inhibited (Figure 2-14A). A double-reciprocal plot of the data showed that there were two distinct populations of α5β1 integrins, one with high affinity, the other with much lower affinity compared with the first group to fibronectin (Figure 2-14B). In the presence of bacitracin the maximum binding capacity of both groups was greatly decreased.

If bacitracin behaves as a direct competitive inhibitor of fibronectin, i. e. they compete for the same binding site in integrins, the concentration of bacitracin required for half maximal inhibition of fibronectin binding should increase in parallel with the fibronectin concentration, and the maximal extent of inhibition should be unchanged. However, our results (Figure 2-15) demonstrated that when concentration of fibronectin was increased over 4 fold from 200 ng/ml to 800 ng/ml, the concentration of bacitracin required for half-maximal inhibition of fibronectin binding was changed less than 2 fold. In addition, the maximal extent of inhibition decreased with increasing bacitracin concentration. This result suggests that bacitracin may cause an allosteric inhibition in which bacitracin does not compete directly with ligand for binding to β1 integrin. Instead, it binds to another site on β1 integrin and induces allosteric changes which interfere with ligand binding.

Figure 2-14A

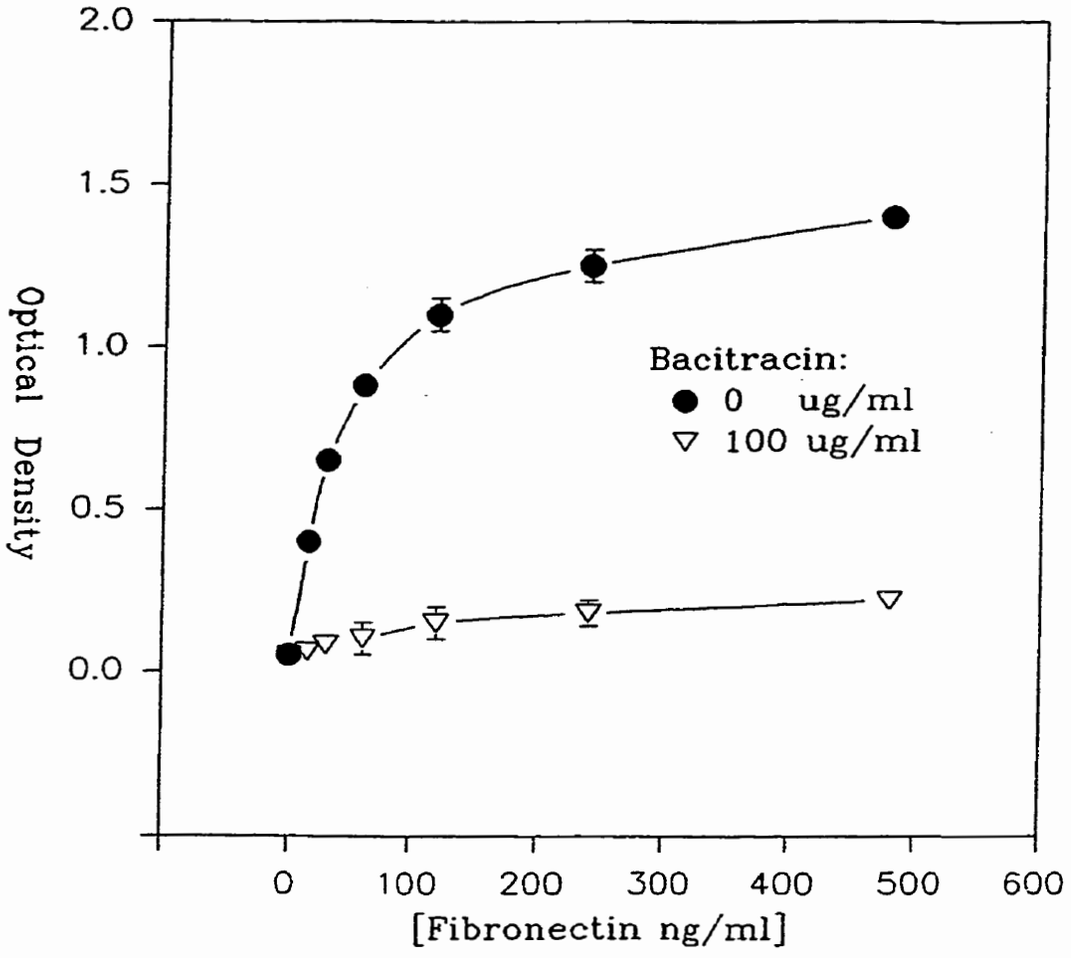

2.0
1.5
1.0
0.5
0.0
Optical Density
Bacitracin:
0 ug/ml
100 ug/ml
0
100
200
300
400
500
600
[Fibronectin ng/ml]

Figure 2-14A: Effect of bacitracin on fibronectin binding to $\alpha 5\beta 1$ integrin. Soluble fibronectin labeled with biotin was incubated with immobilized $\alpha 5\beta 1$ integrin-coated with 96-well plate in the presence of indicated concentration of bacitracin. The level of bound fibronectin was determined following the reaction with alkaline phosphatase-conjugated avidin and substrate.

Figure 2-14B

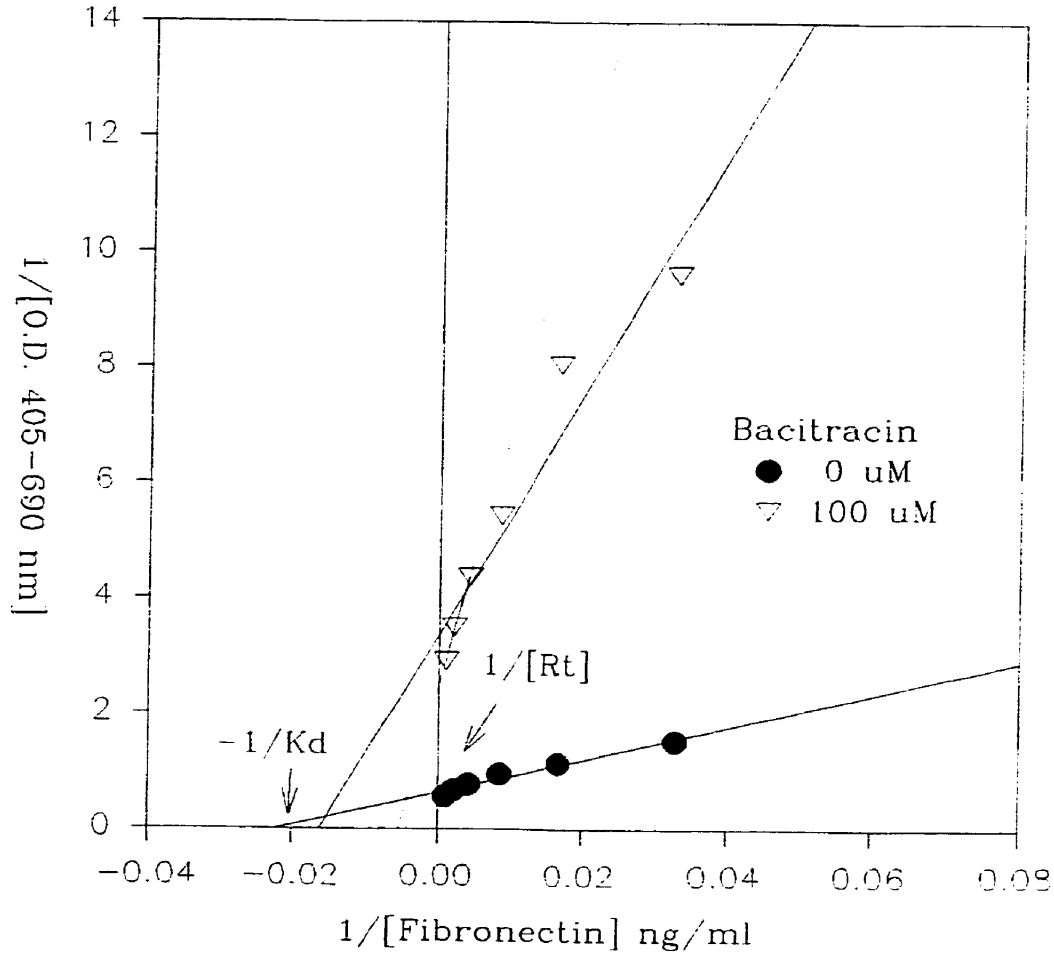

14
12
10
8
6
4
2
0
1/[O.D. 405-690 nm]
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
1/[Fibronectin] ng/ml
Bacitracin
0 uM
100 uM
1/[Rt]
−1/Kd

Figure 2-14B. Double-reciprocal plot of the data shown in figure 2-14A. By linear regression analysis, two distinct classes of $\alpha 5\beta 1$ integrin populations can be distinguished based on their affinity to fibronectin.

Figure 2-15

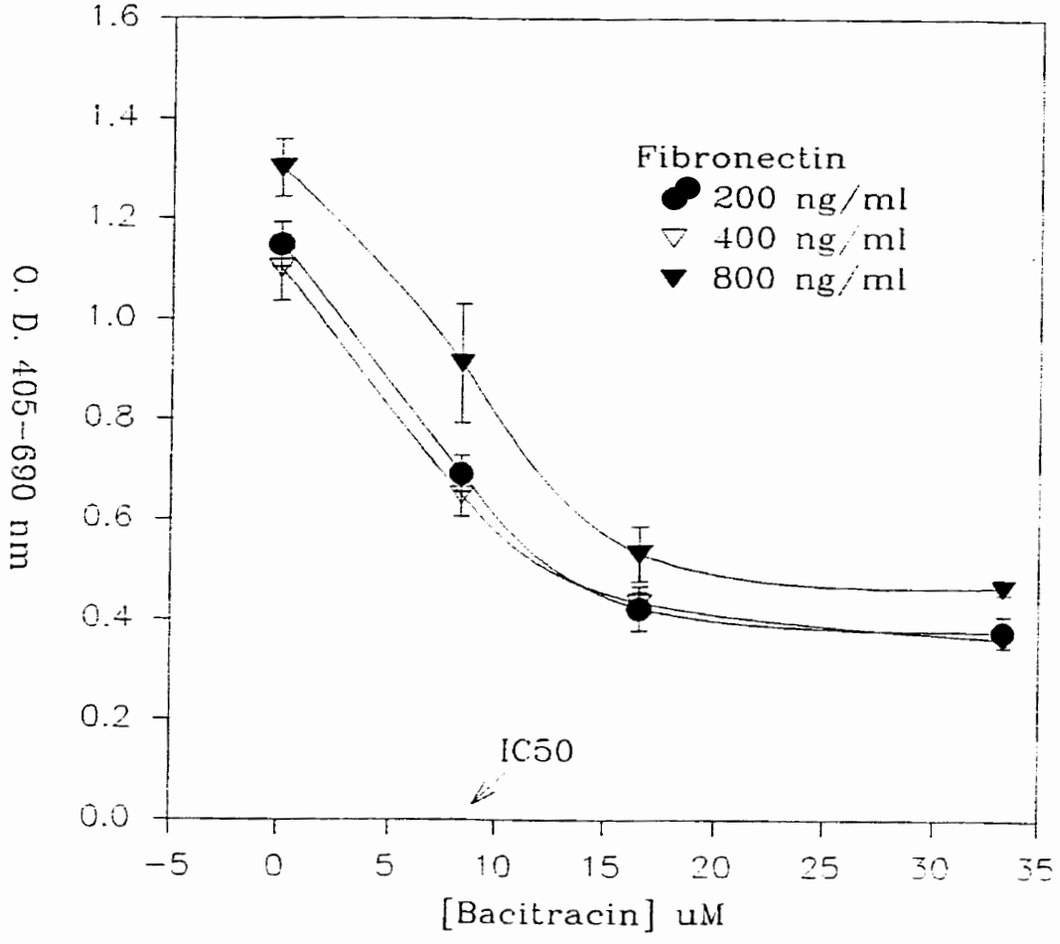

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O. D. 405-690 nm
Fibronectin
200 ng/ml
400 ng/ml
800 ng/ml
IC50
-5
0
5
10
15
20
25
30
35
[Bacitracin] uM

Figure 2-15. Effect of fibronectin concentration on the concentration of bacitracin required for half-maximal inhibition of fibronectin binding to $\alpha 5\beta 1$ integrin. Binding of biotin-labeled soluble fibronectin to immobilized purified $\alpha 5\beta 1$ integrin was measured for a range of concentration of bacitracin at three different fibronectin concentrations.

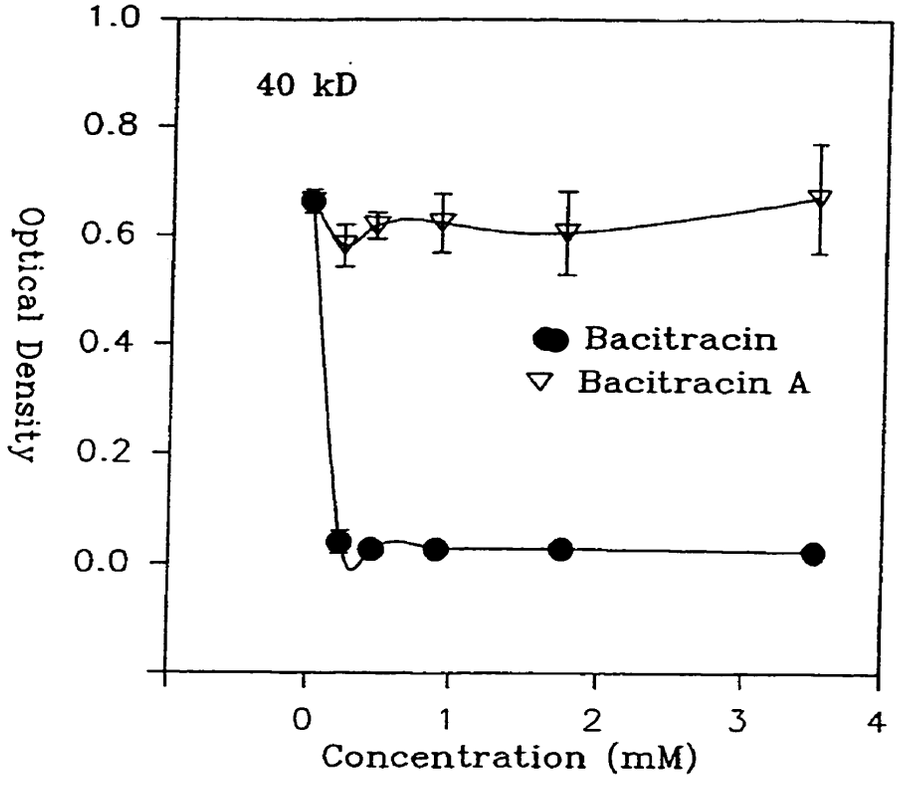
6. Bacitracin A can partially inhibit α5β1 but not α4β1 integrin-mediated cell adhesion.

The commercial bacitracin from Sigma Company is a mixture consisting of more than 20 different bacitracin (Tsuji K and Robertson JH, 1975). However, bacitracin A and bacitracin B account for more than 60% of the material (Medina V et al, 1993). In order to further identify which components account for the inhibitory activity, HPLC-purified bacitracin A was tested for its ability to inhibit α4β1 and α5β1 integrin-mediated cell adhesion. Jurkat cells use α4β1 integrin to bind 40 kD fibronectin fragment and α5β1 to bind to 120 kD fibronectin fragment (Ruoslahti E and Pierschbacher MD, 1986; Wayner EA et al, 1989). As shown in figure 2-16, α4β1 integrin-mediated Jurkat cell adhesion to 40 kD fibronectin fragment was not influenced even at 3.5 mM bacitracin A, while this concentration of bacitracin has previously been shown to inhibit adhesion by more than 90%. α5β1 integrin-mediated cell adhesion to 120 kD fibronectin could be partially inhibited by bacitracin A. The inhibition reached maximum plateau of about 30-40% of the total cell adhesion at 1 mM of bacitracin A (Figure 2-16).

7. Bacitracin does not change β2 integrin expression level and CLIBS expression

Monoclonal antibody 24 (mAb24) reacts with a common epitope on αL, αM and αX subunits which can combine with β2 subunits to form αLβ2, αMβ2 and αXβ2 (Dransfield I and Hogg N, 1989). The expression of this epitope was found to parallel ligand binding activity of these integrins (Dransfield I and Hogg N, 1989). In order to test whether bacitracin can cause changes of αLβ2 integrin expression level and functional state, JY cells were treated with 3.5 mM bacitracin and then tested for αLβ2 expression

Figure 2–16


40 kD
Optical Density
1.0
0.8
0.6
0.4
0.2
0.0
Bacitracin
Bacitracin A
0
1
2
3
4
Concentration (mM)

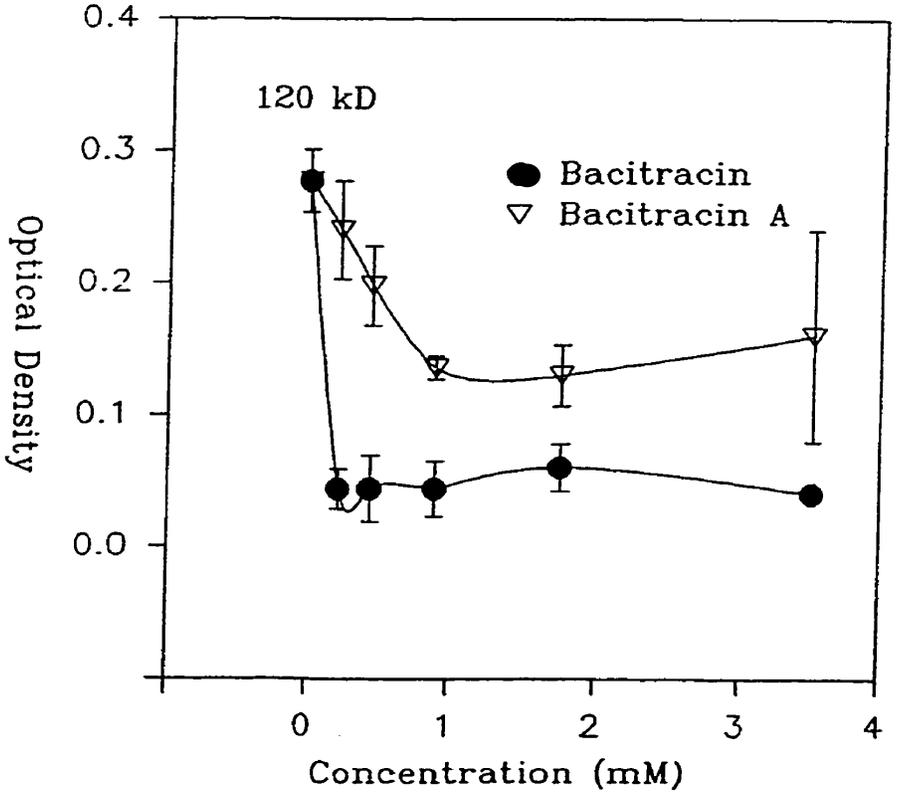
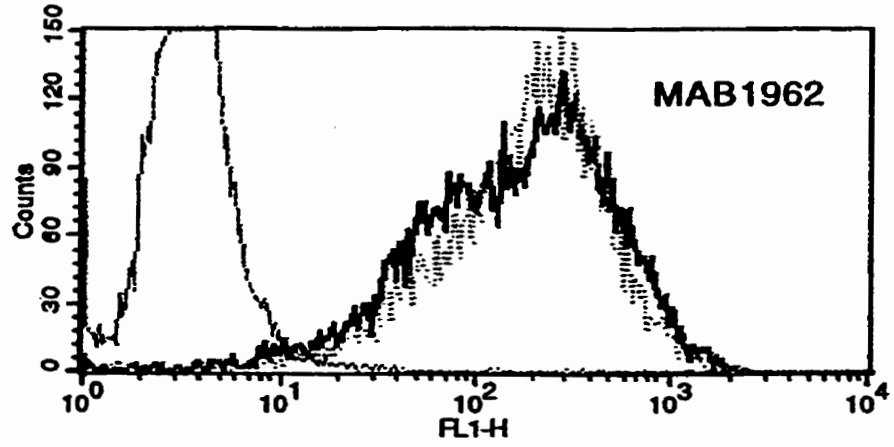

120 kD
Optical Density
0.4
0.3
0.2
0.1
0.0
Bacitracin
Bacitracin A
0
1
2
3
4
Concentration (mM)

Figure 2-16: Effects of bacitracin A on $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin-mediated cell adhesion. Jurkat cells were treated with indicated amounts of bacitracin A or bacitracin at $37^{0}C$ for 30 min. The cells were then assayed for adherence to 40 kD or 120 kD fibronectin fragments in the presence of bacitracin A or bacitracin. The results for all experiments are the mean of sextuplicate assays that have been repeated at least two times.

level and functional state by mAb24. Untreated JY cells expressed the epitope recognized by mAb24 (Figure 2-17). This was consistent with the observation that αLβ2 integrins on JY cell were already activated and could mediate spontaneous homotypic aggregation (Rothlein R and Springer TA: 1986). Pretreatment of the cells with PMA or bacitracin did not cause changes in the expression of the epitope recognized by mAb24, indicating that bacitracin did not induce obvious changes of αLβ2 integrin functional state. The expression of epitope recognized by another monoclonal antibody against β2 integrin (MAB1962) did not change either under the treatment of PMA and bacitracin, suggesting that bacitracin changed neither the expression level of αLβ2 integrin nor the functional state of αLβ2 on cell surface.

8. Protease activity in bacitracin and integrin-mediated cell adhesion:

Bacitracin has been shown to inhibit α5β1 integrin-mediated cell adhesion to 120 kD fibronectin fragment (Table 2-2), but enhance αvβ3 integrin-mediated cell adhesion to 120 kD fibronectin fragment (Figure 2-4). This suggests that the mechanisms that bacitracin affects on these two integrin-mediated cell adhesion may be different. Since bacitracin can inhibit soluble fibronectin binding to purified α5β1 integrins (Figure 2-11), we were curious about the effects of bacitracin on soluble fibronectin binding to purified αvβ3.


MAB1962
Counts
0
30
60
90
120
150
10^0
10^1
10^2
10^3
10^4
FL1-H

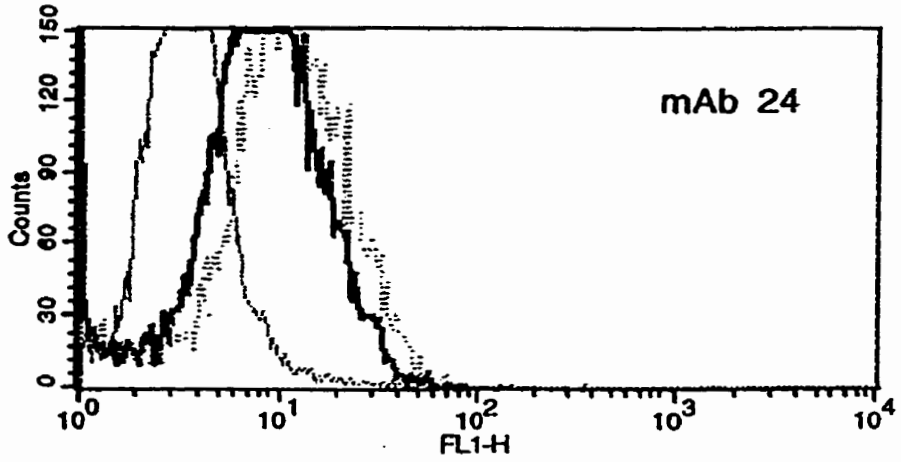

mAb 24
Counts
0
30
60
90
120
150
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 2-17. The effect of bacitracin on β2 integrin expression levels and conformation. JY cells were incubated with the indicated antibodies in the absence (solid dark line) or presence (dashed line) 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 antibody mAb 24 and MAB1962 in the presence of bacitracin are totally superimposed on their corresponding untreated counterparts.

8.1 Bacitracin effects on soluble fibronectin binding to immobilized αvβ3 integrin

Biotin-labeled soluble fibronectin was reacted with purified αvβ3 integrin coated on plates in the presence of bacitracin. In contrast to cell adhesion assay, bacitracin could inhibit soluble fibronectin binding to αvβ3 integrin (Figure 2-18). This result suggests other factor(s) may also influence the interaction between αvβ3 and fibronectin.

8.2 The inhibitory effects of bacitracin on integrin-mediated cell adhesion are not completely mediated by the protease activity of component(s) in bacitracin

After communicating with Dr. L. Kesner (Department of Biochemistry, SUNY-Health Science Center at Brooklyn, Brooklyn NY), we were told that the component(s) in bacitracin might contain protease activity, which was sensitive to protease inhibitor PMSF. To test this, fibronectin was incubated with 3.5 mM bacitracin at 37^{0}C for 2 hours in the presence or absence of 1 mM PMSF. The samples were used for 8% SDS-PAGE in reduced condition. Bacitracin at 3.5 mM could digest fibronectin and this effect could be blocked by 1 mM PMSF (Figure 2-19).

In order to roughly quantitate how much protease activity in bacitracin contributes to the inhibitory effects of bacitracin on integrin-mediated cell adhesion, bacitracin was pre-incubated with 1 mM PMSF at 37^{0}C for 30 min, and then was used in integrin-mediated cell adhesion assays. Cells in the presence of 1 mM PMSF were used as control. As shown in Figure 2-20, in the presence of bacitracin pretreated with 1 mM PMSF, around 30% α5β1 integrin-mediated cell adhesion to fibronectin could be inhibited by bacitracin, while PMSF control had almost no effects on cell adhesion.

Figure 2-18

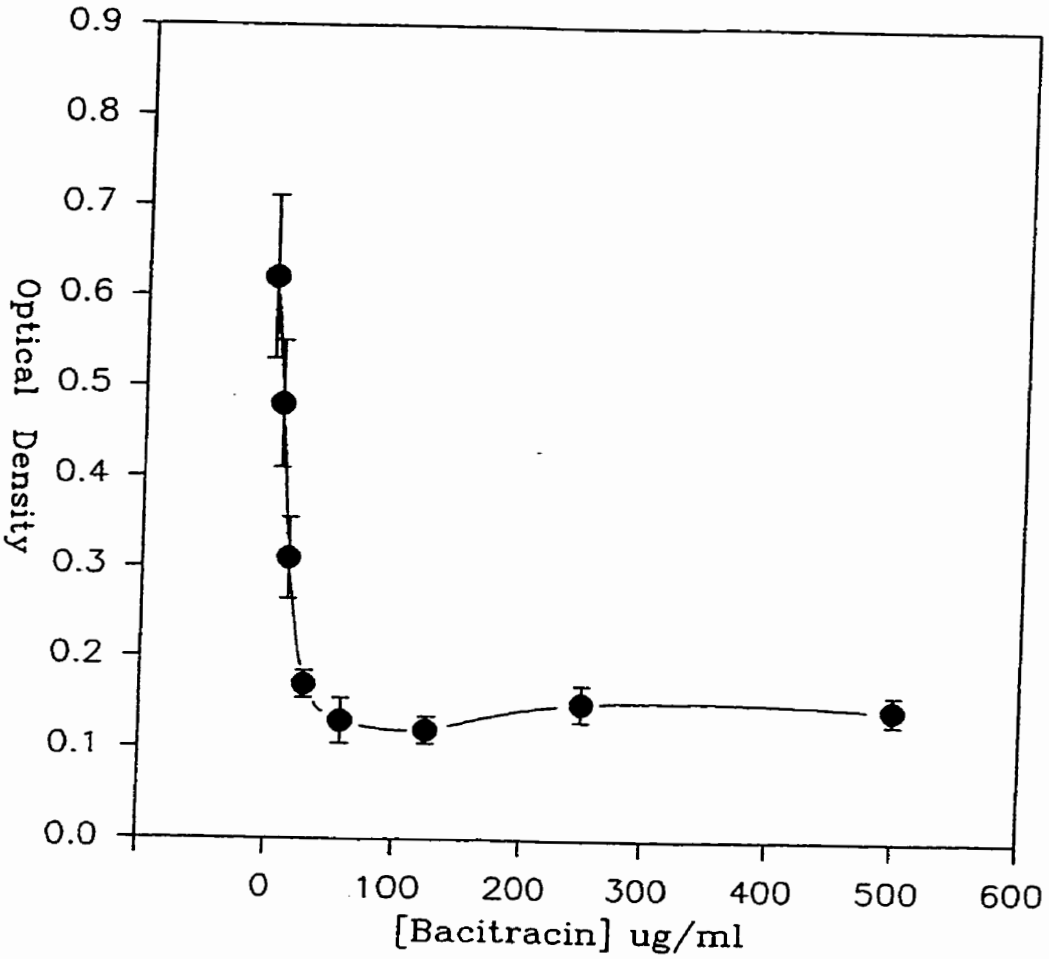

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical Density
0
100
200
300
400
500
600
[Bacitracin] ug/ml

Figure 2-18: The effects of bacitracin on soluble fibronectin binding to immobilized purified αvβ3 integrin. 200 ng/well purified αvβ3 integrin from human placenta was coated on 96-well plate. Biotin-labeled soluble fibronectin was reacted with immobilized αvβ3 integrin in the presence of indicated amount of bacitracin. The level of bound fibronectin was determined at 405 nm following reaction with alkaline phosphatase conjugated avidin and substrate.

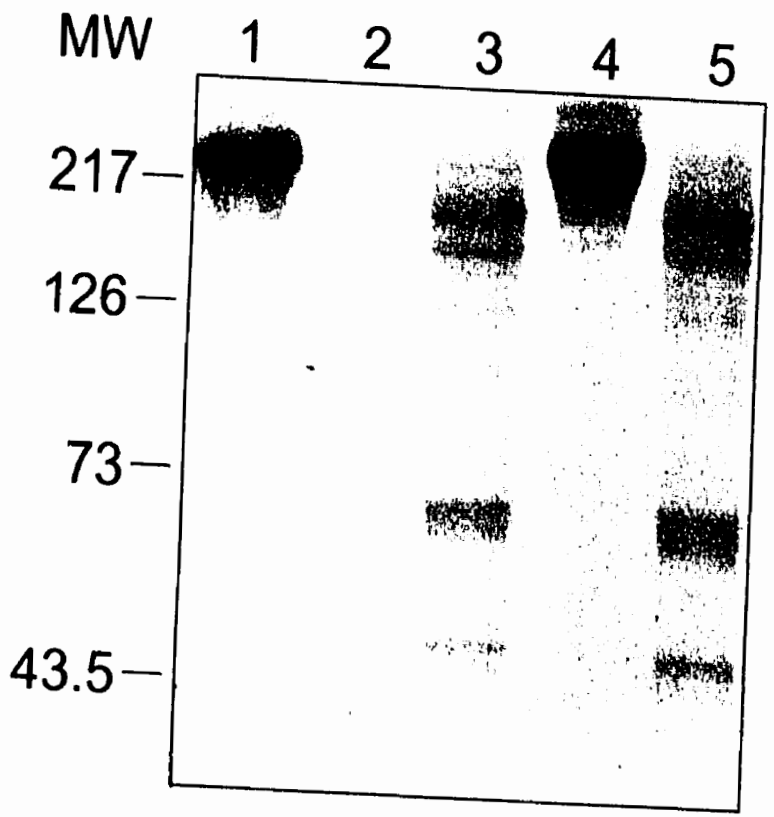

MW
1
2
3
4
5
217
126
73
43.5

Figure 2-19: The effects of protease activities in bacitracin on fibronectin. 3.5 mM of bacitracin (Lane 2) was pretreated (Lane 4) or not (Lane 3) with 0.5 mM PMSF at 37^0C for 10 min then incubate with fibronectin at 37^0C for 2 hours. Bacitracin untreated fibronectin (Lane 1) and fibronectin treated with 3.5 mM of bacitracin which was pretreated with the same amount of alcohol as in PMSF (Lane 5) were used as control. All the samples were loaded in 8% SDS-PAGE in reduced condition. Molecular weight markers are labeled on the left.

Figure 2–20

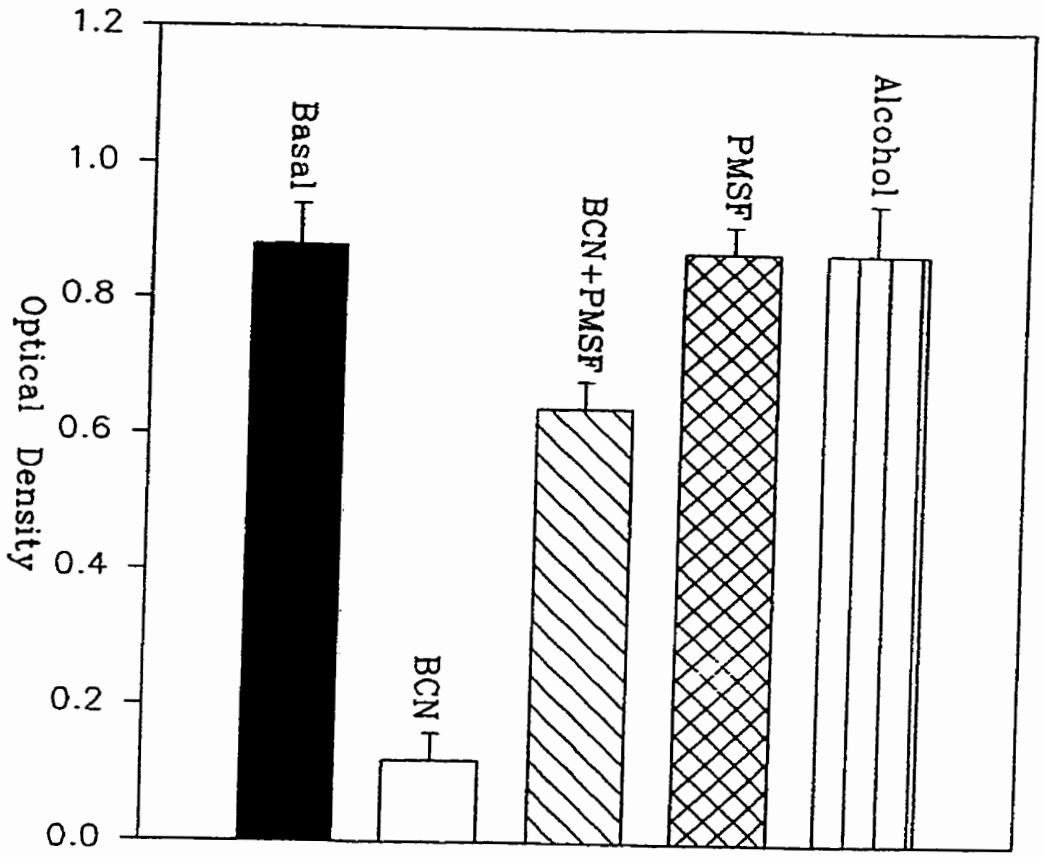

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density
Basal
BCN
BCN+PMSF
PMSF
Alcohol

Figure 2-20: The effects of bacitracin on $\alpha 5\beta 1$ integrin-mediated cell adhesion in the presence of PMSF. Jurkat cells were incubated with bacitracin pretreated by 1 mM PMSF at 37^0C for 10 min. The cells were used for cell adhesion assay to fibronectin. The results for all experiments are the mean of sextuplicate assays that have been repeated at least two times.

These results suggested that the inhibitory effects of bacitracin to $\alpha 5\beta 1$ integrin-mediated cell adhesion were not completely contributed to the protease activities in bacitracin.

Thus, we concluded that components in commercial bacitracin contain protease activities. However, bacitracin induced inhibitory effects on integrin-mediated cell adhesion is not completely related to the protease activities in bacitracin, which can interfere cell adhesion to integrin ligands.

Discussion

The present research was initiated to examine the possible role of protein disulfide isomerase-like activity in the control of integrin-mediated cellular adhesion. This hypothesis was proposed based on the observations that (1) reduction of disulfide bonds in integrins can regulate their functional activity, and (2) protein disulfide exchange mechanisms have been identified on the surface of lymphocytes. The significant findings of this research are (1) Cell surface PDI activity is not involved in the regulation of integrin function. (2) Peptide antibiotic bacitracin exhibits inhibitory effects on β1, β2 and β7 but less extent to β3 integrin-mediated cell adhesion. The mechanisms by which bacitracin inhibits β1 integrin-mediated cell adhesion is not due to the reduction of β1 integrin expression level, but may cause allosteric inhibition of integrin function. (3) Some of the PDI inhibitors such as tocinoic acid and somatostatin can induce β1 integrin conformational changes, leading to the exposure of CLIBS.

1. Role of PDI-like activity in the regulation of integrin function.

1.1 PDI activity and integrin functional regulation

PDI exhibits a broad specificity in catalyzing the folding of variety of proteins. During this process, PDI functions as a catalyst of intramolecular disulfide bond formation (Novia R and Lennarz WJ, 1992). Therefore, this research focus on the possible role of PDI in integrin functional regulation through disulfide bond exchanges in integrin.

The roles of disulfide bonds of integrins in integrin functional regulation are unclear. However, several studies have shown that they may be critical for integrin functional regulation. First, β subunits of the integrin family have 56 highly conserved cysteine residues, these are conserved from Drosophila to humans, in the extracellular domain of the molecules. These cysteine residues are proposed to form 28 nonconsecutive disulfide bridges (Calvete JJ et al, 1991; Bogaert T et al, 1987; DeSimon DW and Hynes RO, 1992). Second, integrin activation is accompanied by integrin conformational changes near integrin disulfide bonds in β1 (Ni, HY et al, 1998a) and β3 integrin subunits (Honda S et al, 1995). Furthermore, the bifunctional reducing agent DTT can activate both purified integrins (Davis GE and Camarillo CW, 1993) and integrins on cell surface (Ni HY et al, 1998a). In addition, integrin-ligand interaction causes the disulfide chain exchanges in integrin (Gofer-Dadosh N et al, 1997). All these data suggest possible roles of integrin disulfide bonds in the regulation of integrin functions.

Protein disulfide isomerase is a resident protein in the endoplasmic reticulum but it can also be found on the surface of various cell types including lymphocytes (Tager M et al, 1997). PDI reaches the surface as the result of an "overflow" from the ER (Yoshimori T et al, 1990). Surface PDI is thought to be able to exert reductive functions (Mandel R et al, 1993). Therefore, all of these observations raise the possibility that PDI activity may have a regulatory function for integrin.

1.2 PDI inhibitors do not interfere with integrin function.

Cell surface PDI activity has been reported to be effectively inhibited by a membrane-impermeant sulfhydrl blocker-DTNB (Mandel R et al, 1993), synthetic peptides somatostatin and tocinoic acid (Essex DW et al, 1995), and monoclonal antibody (RL 77) against PDI (Mandel R et al, 1993) in different systems. However, all these PDI inhibitors exhibited no effects on β1 integrin-mediated cell adhesion in this research. The concentrations of all these PDI inhibitors used in the study exceeded or were equal to those reported to effectively inhibit cell surface PDI activity (Mandel R et al, 1993; Mandel R et al, 1993; Essex DW et al, 1995). Moreover, 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). Therefore, the results of our studies do not support a role for conventional PDI activity in the regulation of β1 integrin-mediated cell adhesion.

However, our argument of the role of conventional PDI in the regulation of integrin-mediated cell adhesion is contradictory to several papers which show the possible roles of PDI in the regulation of αIIbβ3 integrin function in freshly purified platelets (Chen K et al, 1992; Essex DW et al, 1999; Lahav J et al, 2000). Protein disulfide isomerase can be released from activated platelets (Chen K et al, 1992). The secreted PDI can mediate platelet aggregation by activating GPIIbIIIa integrin and the secretion parallels with platelet aggregation. The platelet aggregation can be blocked by adding reduced denatured ribonuclease A, a competing substrate for PDI, but not native ribonuclease. Anti-PDI Fab fragments inhibit activation of GPIIbIIIa when added before, but not after platelet activation (Essex DW et al, 1999). Recently, Lahav J et al show that

αIIbβ3 integrin and α5β1 integrin-mediated cell adhesion in freshly purified platelets can be inhibited by both PDI inhibitor bacitracin and functional-blocking antibodies to PDI (Lahav J et al, 2000). Therefore, GPIIbIIIa integrin is regarded as the target for PDI in the activated platelets. This is completely different from our conclusion that PDI is not involved in the functional regulation of β1 integrins in IL-2 dependent peripheral lymphocytes.

The contradictory results may be explained if one considers integrins with different activation states in different experimental systems. Integrins on freshly purified platelets are completely in their resting state while integrins on IL-2 dependent peripheral lymphocytes are already in their active state because of the persistent stimulation of IL-2 (Pankonin G et al, 1992; Umehara H et al, 1994). Resting integrins and active integrins are different in the number of free Cys residues in the β subunits. The former has 2-3, while the latter has 4-5 unpaired Cys residues. Modifying these unpaired Cys residues in resting integrins will prevent these integrins from becoming activated by DTT. However, modifying unpaired Cys residues in active integrins will not change their ligand binding function (Yan B and Smith JW, 2000). Therefore, bacitracin or inhibitory anti-PDI antibodies may modify free Cys residues in the resting integrins on platelets or active integrins on IL-2 dependent peripheral lymphocytes by inhibiting membrane PDI activity. This will cause different results if these integrins are stimulated later on. In addition, the conclusion of the role of PDI on β1 regulation is only based on the conventional form of PDI. If PDI with mutations or if different form(s) of PDI exists on the cell surface, this could alter our conclusion. For example, PDI with mutations in mAb RL77 recognized epitope will abolish the inhibitory function of mAb RL77 to PDI.

Furthermore, this research only considered the roles of PDI in the functional regulation of integrins. Whether other proteins with functional homology to PDI may exist on cell surface are not considered in this system.

1.3 Isomerase activities of integrin ligand fibronectin and integrin beta subunits

Recently, fibronectin has been identified to have PDI activity (Langenbach KJ and Sottile, 1999). Multimeric fibronectin is a major constituent of the extracellular matrix. The ability of fibronectin to be converted to monomeric fibronectin by treatment with disulfide-reducing agents suggests that the extracellular matrix fibronectin is stabilized predominantly by inter- or intramolecular disulfide exchange (Mckeown-Longo PJ and Mosher DF, 1983; Ali IU and Hynes RO, 1978). Fibronectin has been demonstrated to have PDI activity. The PDI activity of the fibronectin is localized to the twelfth type I module of fibronectin (I_{12}). I_{12} contains a C-X-X-C motif that is thought to be the same sequence in the active sites of PDI and thioredoxin (Luz JM and Lennarz WJ, 1996). This isomerase activity appears to be partially cryptic because partial removal of the I_{10}-I_{12} regions will increase its isomerase activity (Langenbach KJ and Sottile J, 1999). The isomerase activity in fibronectin may be very important in cross-linking of fibronectin in the extracellular matrix. The cryptic nature of fibronectin's protein-disulfide isomerase activity indicates that this isomerase activity can be highly regulated by conformational changes of fibronectin. Fibronectin itself and the environment may have the ability to regulate the activity of its isomerase. Conformational changes have been detected in fibronectin following binding of fibronectin to surfaces (Fukai GF et al, 1995; Ugarova TP et al, 1995) or following alterations in pH and ionic strength

(Markovic Z et al, 1983; Williams EC et al, 1982). Conformational alterations in fibronectin have also been shown to lead to exposure of cryptic binding sites (Morla A and Ruoslahti E, 1992; Hocking DC et al, 1994).

The role of isomerase activity of fibronectin in integrin-fibronectin interaction is unclear. However, integrin β subunits contain 56 conserved cysteines, and the interaction between integrin and fibronectin induce the exposure of CLIBS near predicted disulfide bond pairs. One possibility is that cells may use α5β1 integrin to adhere to RGD sequence located in the 10^{th} of the homologous repeating units in fibronectin, and then the isomerase activity in fibronectin and possibly in β1 integrin (discussed later) cause the intrachain, or interchain disulfide bond exchanges, which results in firm adherence. Therefore, there is no reason to neglect the isomerase activity of fibronectin in the integrin-fibronectin interaction. It is unknown whether PDI inhibitors such as tocinoic acid, somatostatin and mAb RL77 can inhibit isomerase activity of fibronectin. However, there is no evidence to indicate that fibronectin has peptide binding sites such as the one in PDI. Since fibronectin and PDI are highly different in their primary and conformational structure, the chance that these PDI inhibitors inhibit isomerase activity of fibronectin seems very low. The cryptic nature of fibronectin's protein-disulfide isomerase activity may also make it more difficult for DTNB to access the isomerase functional regions of fibronectin. It is unknown whether other integrin ligands besides fibronectin also have PDI activities.

Integrin β subunits may also have PDI activity. Integrin β subunits contain C-X-X-C sequences in their extracellular domain (Erle DJ et al, 1991). Two of them are located in the N-terminus, the other seven in the cysteine-rich region, near the

transmembrane area of the extracellular domain. These C-X-X-C regions are highly conserved among β subunits (Calvete JJ et al, 1991; Bogaert T et al, 1987). Since C-X-X-C region is considered as the isomerase functional site in PDI and fibronectin, it is reasonable to question whether integrin β subunits have isomerase activity. Recent results from O'Neill S et al have demonstrated that both integrin β1 and β3 subunits have PDI-like activity, and the PDI-like activity in β3 subunit is much higher than in β1 subunit (O'Neill S et al, 2000). What the roles of PDI-like activity in integrin β subunits play in integrin-ligand interaction are unknown? In fact, in the process of testing for ligand-induced change in the affinity of platelet α2β1 to collagen, Gofer-Dadosh et al reported that when viable platelets were passed through a column of fibrillar collagen, a high affinity fraction of α2β1 left on the collagen could be eluted with DTT and 2% SDS but not EDTA, chaotropic agents or low pH (Gofer-Dadosh N et al, 1997). This result suggests that ligand binding to the integrin induces the formation of a new intrareceptor disulfide bond in a fraction of α2β1 integrins (but not inter-receptor disulfide bond because fibrillar collagen does not contain cysteine), which increases the affinity of α2β1 integrin to fibrillar collagen. Thus, it seems that during integrin-ligand interactions, disulfide bond exchanges occur. However, which protein provides isomerase activity is unknown. Both integrin β subunits and PDI can be the candidates.

1.4 Role of DTT and disulfide bonds in integrin functional regulation

The role of integrin intrachain disulfide bonds in the regulation of integrin function is unclear. Although data from several sources suggest that disulfide bonds may

be important for the functional regulation, there is still little direct data to support this idea. Furthermore, some results appear to contradict this concept.

The idea that intrachain disulfide bonds may be involved in the regulation of integrin functions is largely based on the effects of DTT on integrin activity. DTT, a bifunctional reducing agent has been reported to be able to activate cell adhesion mediated by β1 integrin (Ni HY et al, 1998a), β2 integrin (Edwards BS et al, 1995) and β3 integrin (Peerschke EI et al, 1995) in several different systems. In all these systems, intact cells were treated with DTT and tested for their binding ability to the ligands or for aggregation. Since DTT is a nonspecific reducing reagent, and DTT is cell membrane permeable (Goldman H et al, 1970), it can nonselectively interact with disulfide bonds in all the proteins inside the cells and on cell surface. Thus it can not guaranteed that any changes in integrin behavior are the direct effects of DTT on integrins. Furthermore, DTT may also act through additional pathways to induce adhesion. Edwards BS et al found that DTT activated LFA-1 in HSB2 T cells without detectable disulfide reduction in LFA-1 αL or β2 chains immunoprecipitated from these cells. Moreover, DTT treatment of NK cells did not hinder binding of KIM127 and KIM185, monoclonal antibodies which recognize epitopes in the potentially DTT-susceptible cysteine-rich domain of the β2 chain (Edwards BS et al, 1995). We have tested the exposure of free –SH groups on integrins upon activations by Mn++ and DTT in both Jurkat cells and IM9 cells in an attempt to answer whether β1 integrin-mediated cell adhesion results from breaking disulfide bonds of β1 integrin. Our results show that Jurkat cells treated with 10 mM DTT can greatly increase the amount of β1 integrin free –SH groups, but β1 integrin-mediated cell adhesion has no further increase. In contrast, 2 mM Mn++ treated IM9 cells

exhibited an increased binding ability mediated by β1 integrin without changes in free –SH groups (Ni HY, Ph. D thesis). Thus, all these results could suggest that direct biochemical modification of integrins by DTT is not involved. Instead, signaling-associated enzymatic activities may be involved in DTT activation of integrins.

The only direct evidence to support the idea that disulfide bonds in integrin may be involved in the integrin functional regulation comes from the results obtain by Davis GE and Camarillo CW. They coated microwell plates with purified α5β1 integrins from HL-60. In the presence of different concentration of DTT, the binding of 120 kD fibronectin fragment was found to be increased. At 1 mM DTT concentration, the binding level increased by 50% and reached the maximum plateau at 10 mM of DTT. Thus, they concluded that DTT acted in part by directly influencing the integrin-ligand interaction and DTT might change the conformation of α5β1 through disulfide bond reduction in a way that favors fibronectin binding (Davis GE and Camarillo CW, 1993). However, the effect of DTT on 120 kD fibronectin fragment disulfide bonds, especially isomerase activity is not considered in their conclusion.

Integrin β subunit contains presumed large loops formed by the linkage between cysteines, for example, Cys7-Cys444 and Cys415-Cys671 in β1 integrins. Activation of β1 integrins leads to the exposure of epitopes along these large loops (Ni NY et al, 1998a; Honda S et al, 1995). In an attempt to further characterize the role of these long-range disulfide bonds in integrin functional regulation, a point mutation that changes Cys655 into Tyr655 in GPIIa was made. Cys655 is thought to participate in the formation of a long-range disulfide bond with Cys406 (Calvete JJ et al, 1991). When mutant GPIIIa cDNA is transfected into CHO cells with GPIIb, it is capable of forming a complex with

GPIIb on the cell surface. This integrin complex can bind to RGD-containing peptides and undergo conformational changes similar to the wild type (Wang R, et al, 1997). These data suggest that the mutation at the highly conserved cysteine residue 655 of GPIIIa subunit has no effect on integrin function. In contrast, mutation in Cys 374 into Tyr374 of GIIIa in Glanzmann thrombasthenic patient leads to a defect in surface expression of GPIIb-IIIa on the patient's platelets (Grimaldi CM et al, 1996). Mutations in Cys435 into Ala435 or Cys5 into Ala5 cause integrin constitutive activation (Liu CY et al, 1998; Yan B et al, 2000). Thus, the role of integrin intrachain disulfide bonds in the regulation of integrin functions needs further exploring.

Therefore, integrin intrachain disulfide bonds are undoubtedly important in the regulation of integrin conformation. Whether these conformational changes are related to integrin functional regulation requires further investigation.

2. The possible mechanisms by which bacitracin interferes with integrin-mediated cell adhesion.

Bacitracin displayed an inhibitory effect on β1 integrin-mediated cell adhesion and this inhibitory effect does not appear to be related to the effects on PDI since other PDI inhibitors displayed no detectable effects on β1 integrin-mediated cell adhesion. Bacitracin also shows selective inhibitory effect on integrin-mediated cell adhesion. This property may indicate differential regulatory characteristics of different integrins on lymphocytes, which might be helpful in understanding integrin function-related lymphocyte behavior. Therefore, it is necessary to further examine the mechanism for

such inhibitory effect. However, component(s) in bacitracin also shows protease activity that can influence integrin-mediated cell adhesion. This makes it more difficult to define the possible mechanisms by which bacitracin interferes with integrin-mediated cell adhesion.

2.1 Commercial bacitracin is a heterogeneous product and varies with batch

Bacitracin obtained from Sigma Company is a heterogeneous product containing more than 20 components including different fractions of bacitracin (A-F). Bacitracin A and B account for more than 50% of the total components (Tsuji K and Robertson JH, 1975). However, some of the components are uncharacterized (private communication with Sigma Company). The protease activity in bacitracin has been shown in this research. Protease activity in bacitracin can interfere with integrin-mediated cell adhesion by digesting integrins and/or ligands. Moreover, since bacitracin A and B are the major components in the bacitracin mixture, other components in bacitracin mixture vary from batch to batch. Our result showed that bacitracin A does not completely account for the inhibitory effect of bacitracin on integrin-mediated cell adhesion. Therefore, other component(s) in commercial bacitracin may also contribute to bacitracin-induced inhibitory effect on integrin-mediated cell adhesion.

2.2 The inhibitory effect of bacitracin on integrin-mediated cell adhesion is not completely contributed by protease activity.

Protease in bacitracin can interfere with integrin-mediated cell adhesion by digesting integrin, integrin-associated proteins or integrin ligands. However, based on

FACS data from β1 integrin, in the presence of bacitracin the expression of epitopes located on N-terminal such as N29, JB1A, C-terminal near membrane region of extracellular domain such as B3B11 and conformational epitope 3S3 are unchanged, only B44 epitope expression is increased. Increased B44 epitope expression can also been seen in protease free tocinoic acid and somatostatin-treated cells. This suggests that protease activities in bacitracin may not remove β1 integrin from the cell surface. This is further verified by the result that bacitracin does not reduce the epitope expression on purified β1 integrin coated on plate (data not shown).

Another possibility is that the protease activity in the component(s) of commercial bacitracin may digest integrin ligand, and since different integrin ligands may display different sensitivities to protease(s) in the component of commercial bacitracin, therefore, resulting in different sensitivities to the inhibitory effect of commercial bacitracin. Although we have no evidence to completely exclude this possibility, our data show that (1) In the presence of PMSF, an inhibitor for the protease activity in bacitracin, bacitracin still shows 30-40% inhibitory effect on α5β1 integrin-mediated cell adhesion. (2) Bacitracin A purified by HPLC also displays 30-40% inhibition of α5β1 integrin-mediated cell adhesion, consistent with the result of bacitracin effect on α5β1 integrin-mediated cell adhesion in the presence of PMSF. (3) It has been demonstrated that T lymphocytes use α5β1 integrin to bind to the RGD sequence in 120 kD fibronectin fragment, while RPMI 8866 cells employ αvβ3 to bind the same region in the same fragment. However, bacitracin displays completely different effects on these two integrin-mediated cell adhesions to 120 kD fibronectin fragment, suggesting that this difference is due to the role of bacitracin on integrins or components on cell surface

rather than on ligand. (4) The effect of bacitracin on αvβ3 integrin-mediated cell adhesion to fibronectin is also different from soluble fibronectin binding to αvβ3. Binding of the soluble fibronectin to αvβ3 integrin is inhibited by bacitracin, but the same concentration of bacitracin shows enhancement in αvβ3 integrin-mediated cell adhesion to fibronectin. This result is hard to explain simply by the protease activity in the bacitracin. Therefore, the inhibitory effect of bacitracin on integrin-mediated cell adhesion is not completely a result of the protease activity in the component(s) of commercial bacitracin.

During our experiments, we used PMSF to inhibit protease activity instead of using a mixture containing different kinds of protease inhibitors to remove the influence of the protease activity in bacitracin on integrin-mediated cell adhesion. That is because (1) Bacitracin itself is a potent protease inhibitor. It can inhibit all four groups of protease activities (to be discussed) (Elbein AD, 1983). (2) The protease activity in bacitracin is sensitive to PMSF as shown in Figure 2-19. In the presence of PMSF, the protease activity in bacitracin is almost completely inhibited. (3) Protease-free bacitracin A shows inhibitory effect on α5β1 integrin-mediated cell adhesion (Figure 2-16), consistent with the result in which bacitracin shows inhibitory effect on integrin-mediated cell adhesion in the presence of PMSF. (4) Protease inhibitors may interfere with bacitracin activity or cellular activity (to be discussed).

2.3 Bacitracin effects on integrin and integrin-mediated cell adhesion

2.3.1 Bacitracin causes β1 integrin local conformational changes

Three integrin states have been proposed as a model for the regulation of integrin activity: inactive (I_1), active (I_2) and occupied (I_3) state (Mould AP, 1996). The conformation of these three states appears to be significantly different. Divalent cations tend to shift the equilibrium between I_1 and I_2 in favor of the I_2 state. Removing divalent cations from cation-binding sites on integrins by EDTA will cause the inhibition of integrin functions. Monoclonal antibodies B44, HUTS4, HUST21 and 15/7 are thought to recognize CLIBS on β1 integrin. Expression of CLIBS implies that integrins are maintained in a ligand-occupied state or divalent cation binding state (I_3) which does not mean that the integrins are now being occupied by ligands since increased expression of B44 epitope is observed when peripheral blood lymphocytes cultured on serum-free medium are stimulated by PMA (data not shown). However, bacitracin can induce CLIBS expression on β1 integrin, this suggests that bacitracin can directly induce integrin to change from inactive state (I_1) to ligand occupied state (I_3). This characteristic is quite close to that induced by integrin ligands. Nevertheless, the binding of bacitracin to β1 integrin is a divalent cation independent process, which differs from that of integrin ligand binding. Bacitracin induced integrin β chain conformational changes seems to not be mediated by PDI since inhibitory monoclonal antibody RL 77 to PDI has no effects on β chain conformational changes.

Protein disulfide isomerase has two specific domains (CGHC) with homology to the bacterial protein thioredoxin (CGPC). The CXYC motif was demonstrated to be the thio-disulfide interchange catalytic domain (Freedman RB, 1994; Bardwell JCA, 1994). The antibiotic bacitracin is known to inhibit both the reductive and the oxidative functions of PDI (Roth RA, 1981; Mizunaga T et al, 1990). Recently, fibronectin has

been reported to have PDI activity in twelfth type I module of fibronectin containing a C-X-X-C motif (Langenbach KJ and Sottile J, 1999). Beta-1 integrin has seven C-X-X-C motifs in its primary structure (Erle DJ et al, 1991). Since C-X-X-C motifs in PDI and fibronectin have been shown to catalyze disulfide bond rearrangement, one possible idea is that integrin β chain itself may have disulfide bond rearrangement function. If this idea is true, bacitracin probably directly induces β1 integrin conformational changes by its inhibiting β chain disulfide bond rearrangement functions.

2.3.2 Role of bacitracin A in bacitracin-induced inhibition of β1 integrin-mediated cell adhesion

One of the major components in bacitracin, bacitracin A, shows only 30-40% inhibition of α5β1 but has no effects on α4β1 integrin-mediated cell adhesion, suggesting that α4β1 integrin and α5β1 integrin may use different mechanisms to regulate their functions. In the presence of PMSF bacitracin mixture shows 30-40% of inhibitory effects on α5β1 integrin-mediated cell adhesion. This suggests that bacitracin mixture may have at least 30-40% of the inhibitory effect on α5β1 integrin-mediated cell adhesion regardless of protease activity. However, bacitracin A only contributes to 30-40% of inhibitory effect to α5β1 integrin-mediated cell adhesion. One possibility is that other component(s) in bacitracin mixture other than bacitracin A may also contribute to influence α5β1 integrin-mediated cell adhesion or the inhibitory effect of bacitracin mixture may be the interactive effects of different components in bacitracin.

Since protease-free bacitracin and component bacitracin A-F are not commercially available, it hinders further research on bacitracin effect of integrin-mediated cell adhesion.

2.3.3 Bacitracin effects on αLβ2, α4β7 and αvβ3 integrin

Bacitracin can inhibit αLβ2 integrin-mediated cell adhesion, but doesn't change αLβ2 integrin state as indicated by the expression of epitope recognized by mAb24. Epitopes recognized by mAb B44, HUTS4, HUTS21 and 15/7 also reflect β1 integrin state. However, bacitracin can influence β1 but not β2 integrin state, part of the reason is that mAb24 epitope in β2 integrin in the absence of bacitracin is already expressed at a high level and may not be increased further in the presence of bacitracin.

The effects of bacitracin on β3 and β7 integrin-mediated cell adhesion are different (Figure 2-4). The reason for this difference is unknown at this moment. The possibility that PDI may be involved in this process can not be excluded since PDI activity on platelets has been demonstrated to influence αIIbβ3 integrins activity (Essex DW et al, 1999). We still do not know whether bacitracin can cause allosteric changes by binding directly to other integrins besides β1 integrins. Recently, the cross talk between α5β1 and αvβ3 integrins has been reported (Blystone SD, 1999). Ligation of the integrin αvβ3 integrin inhibits both phagocytosis and migration mediated by α5β1 on the same cell. The mechanisms of such cross talk are thought to be the prevention of α5β1-mediated activation of calcium- and calmodulin-dependent protein kinase II (Blystone SD, 1999). Since both α4β7 and αvβ3 integrin are expressed on RPMI-8866 cells, whether there is any cross talk between them should also be considered. Moreover, the

structural and functional difference between α4β7 and αvβ3 are also important. Differential regulation of chemoattractant-stimulated β3 and β7 integrins activities has been demonstrated (Sadhu C et al., 1998), suggesting that the regulatory mechanisms may greatly differ in β3 and β7 integrin-mediated cell adhesion.

2.4. The possible mechanisms of bacitracin induced inhibitory effects on β1 integrins

2.4.1 Bacitracin may induce allosteric inhibition on integrin function.

The inhibitory effects of bacitracin to integrin-mediated cell adhesion are not related to the changes in the expression level of the integrins on cell surface. Bacitracin can inhibit β1 integrin-mediated cell adhesion regardless of whether integrins on cell surface are activated before or after bacitracin treatment, and bacitracin can inhibit β1 integrin-mediated basal cell adhesion, indicating that bacitracin may act directly on integrins or post-integrin binding events. The direct inhibition of the binding of soluble fibronectin to cells in FACS analysis suggests that the integrins may be the target for bacitracin effects. This result is further supported by the fact that the binding of fibronectin to purified β1 was almost fully inhibited by bacitracin. Moreover, the inhibitory effects of different doses of bacitracin on soluble fibronectin binding to purified α5β1 integrins clarified that bacitracin could induce a steric inhibition.

Double reciprocal plots showed that purified α5β1 integrin when treated with bacitracin could be divided into two populations according to their affinity to soluble fibronectin. Similar observations were obtained by several others in other systems (Diamond MS et al., 1993; Yednock TA et al., 1995; Mould AP et al., 1996).

2.4.2 The interaction site of bacitracin on integrin

The interaction site of bacitracin on integrin is not clear so far. Since bacitracin can induce the expression level of CLIBS on β1 integrin, β1 integrin or both β and α subunit are possibly involved in the interaction with bacitracin. However, the interaction of bacitracin with integrin is divalent cation independent. The divalent cation-binding site of the integrin is located on α subunit of integrin close to integrin ligand binding sites which are located at the N termini of both α and β subunits. Binding of cations to cation-binding sequences in integrin α subunit may directly influence the binding of integrins to their ligands, either by directly contributing to a ligand-binding pocket, or by creating a ligand-binding tertiary structure of the integrin molecule (Horton MA, 1996). However, bacitracin interacts with integrin in a divalent cation independent fashion, reflecting that the binding site of bacitracin in the integrin is possibly different from ligand binding site in the integrin. This is consistent with our conclusion that bacitracin induces allosteric inhibition instead of competitive inhibition. The exact binding site of bacitracin in integrin needs to be investigated further on both primary and conformational structure of integrins.

2.4.3 A model of bacitracin induced effect on integrin function

Based on our data, a model of bacitracin induced effect on integrin function is proposed (Figure 3-1). In this model the ligand-binding site on integrin α and β subunits in inactive state (I_1) are closed. Upon activation, conformational changes near long range disulfide bond and others occur. The ligand-binding site in this situation is opened and ligand-binding pocket is formed (I_2). Binding of integrin ligand, in the presence of

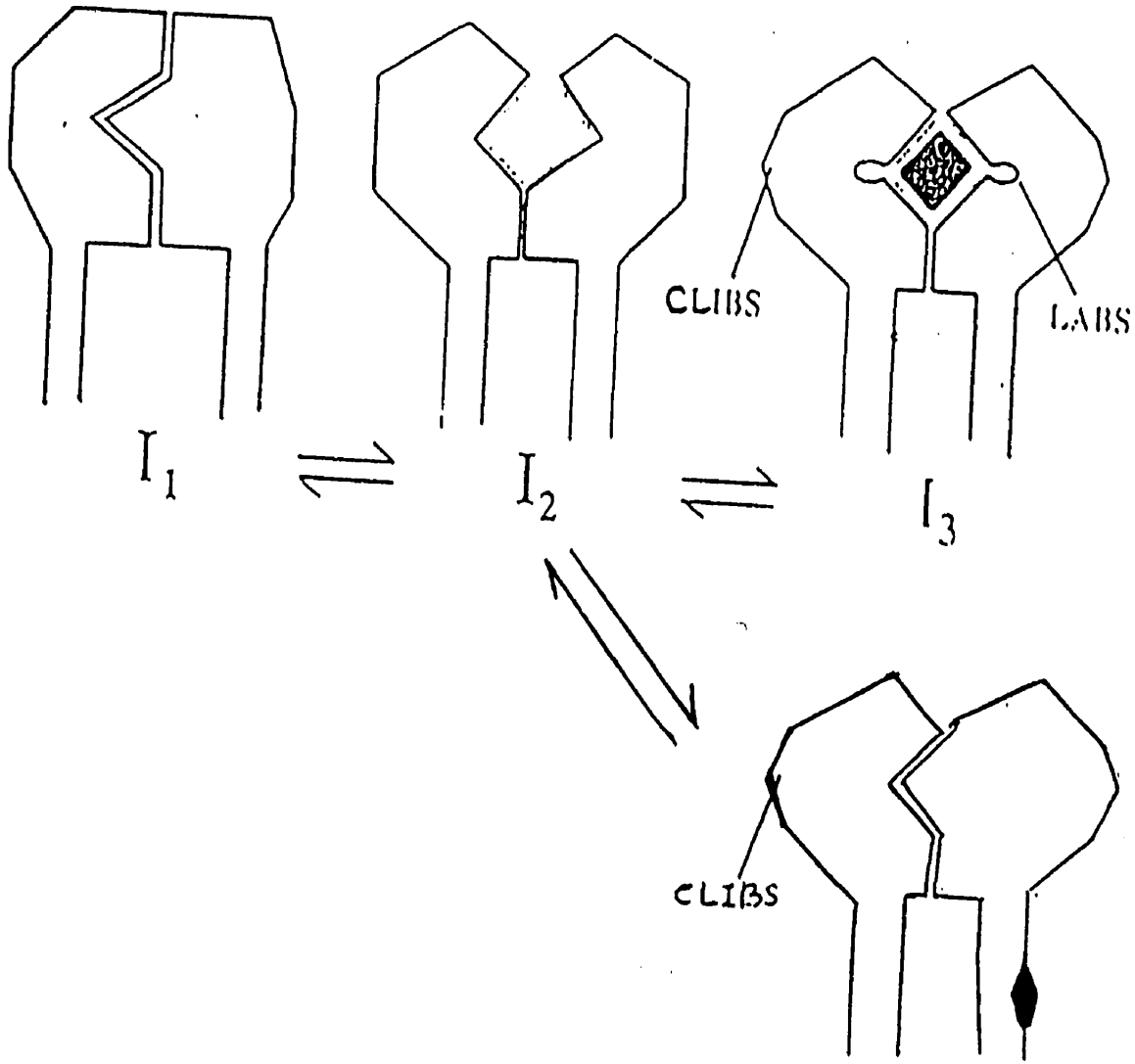

CLIBS
LABS
I_1
I_2
I_3
CLIBS

Figure 3-1: Model of bacitracin effect on integrin. Integrins in latent (I_1) state hide their ligand binding sites. These ligand binding sites are exposed when integrins are activated (I_2) state. Mn^{++} and Mg^{++} shifts the equilibrium between I_1 and I_2 in favor of the I_2 state by binding divalent cation-binding sites, while recognition of ligand by the active integrin causes further conformational changes which lead to the exposure of CLIBS. Binding of bacitracin to integrin may cause similar integrin conformational changes as integrin ligand does. However, the ligand binding sites in integrins are obstructed.

divalent cations, to activated integrin will cause further conformational changes, which lead to firm binding of integrin to ligands and the exposure of CLIBS. However, binding of bacitracin to integrin in divalent cation independent way can induce the same conformational changes as integrin ligands as indicated by the exposure of CLIBS, but such a binding causes the closure of ligand binding site formed by both α and β subunits. The binding site of bacitracin to integrin is not known so far. But it probably is located away from the integrin ligand binding site on both α and β subunits of integrin.

To verify this model, following research work is required: (1) Direct evidence of interaction between integrin and bacitracin. This might be achieved through the use of radioactively labeled bacitracin to demonstrate the interaction of bacitracin with purified integrins. (2) The role(s) of protease activities in bacitracin should be assessed. The commercial bacitracin contains up to 22 different components although bacitracin A and B account for more than 60% in amount of the mixture by weight (Tsuji K, 1975). The protease activities in bacitracin can interfere integrin-ligand interaction by simply degrading integrin or ligand (protease activities in bacitracin will be discussed later). (3) Increased exposure of B44 epitope is related to the direct interaction between integrin and bacitracin. However, detecting the effects of bacitracin on B44 epitope expression using purified β1 integrin can not find any changes in B44 epitope expression (data not shown). The reason is probably due to the fact that in purified β1 integrin the amount of B44 epitope expression is the same as 3S3 while B44 epitope expression on cells account for 25% of 3S3, which is the highest amount of all tested epitope so far (data not shown). This suggests that there are conformational differences between purified β1 integrin and

β1 integrin on cell surface. Purified integrins expressing such high levels of B44 epitope can probably no longer increase B44 epitope expression further.

2.4.4 Caveats to analysis of bacitracin effects

The interaction between integrin and bacitracin may not be as simple as illustrated. Protease activities and other activities in bacitracin make the case more complicated.

2.4.4.1 Protease(s) and integrin function

As already discussed above that protease(s) activity can digest integrin ligands and therefore, interferes with integrin-mediated cell adhesion. On the other hand, the protease(s) and the integrins are functional associated. First, protease(s) in the outside of the cells can influence integrin functions by binding to integrins near ligand recognition site(s) in integrins. For example, αvβ3 integrin has been identified as a potentially critical component for docking matrix metalloproteinase (MMP)-2 at the tumor cell surface (Brooks P et al, 1996; Deryugina E et al, 1997). MMP-2 apparently binds near or to the ECM recognition site(s) of αvβ3 through the COOH-terminal domain of the enzyme. The binding of MMP-2 to integrins could localize ECM degradation to the tumor cell's microenvironment and thus spatially regulate the activity of the enzyme. Second, integrin activation can cause release of proteases. Khan K and Falcone DJ demonstrated that, when activated by collagen IV, fibronectin, laminin and tenascin, cells expressed a 92 kD matrix metalloproteinase (MMP-9) in a dose- and time-dependent manner (Khan K and Falcone D, 1997). Third, matrix metalloproteinase can activate pro-interleukin-1

(Molineaux SM et al, 1993) and release cell surface tumor necrosis factor, tumor necrosis factor receptor (Mullberg J et al, 1995; Kayagaki N et al, 1996; McGeehan G et al, 1994). Moreover, the matrix degradation caused by matrix metalloproteinase can lead to release and/or activation of matrix-bound growth factors, which can profoundly influence integrin functions (Naldini L et al, 1995). However, we don't know what kind(s) of protease exist in bacitracin, whether the protease(s) in bacitracin can bind to integrins and whether this binding (if there is) can influence integrin-ligand interaction.

The protease inside of the cells can also influence integrin-mediated cell adhesion. A calcium-dependent protease calpain, a cysteine protease, is reported to be localized to focal adhesions and cleaves many focal adhesion-related proteins including integrin receptors, focal adhesion kinase, and talin (Du X et al, 1996; Cooray P et al, 1996; Inomata M et al, 1996). Calpain was shown to inhibit both β1, β2 and β3 integrin-mediated cell migration and calpain inhibition specifically stabilizes peripheral focal adhesions, increases adhesiveness and decreases the rate of cell detachment (Huttenlocher A et al, 1997; Stewart MP et al, 1998). The mechanisms that calpain influence integrin-mediated cell function is that calpain cleavage integrin cytoplasmic domain during integrin activation (Du X et al, 1996). Five cleavage sites have been identified. Four of these sites flank two NXXY motifs (Du x et al, 1996). Among integrin β1A, β1D, β2, β3 and β7 subunits tested, the susceptibility to calpain cleavage is common to these integrin β subunits (Pfaff M et al, 1999). Since NXXY motif is critical to the bidirectional signaling functions of integrins and their association with the cytoskeleton, calpain cleavage of the cytoplasmic domain of integrin β subunit cytoplasmic domain provides a means to regulate integrin signaling functions. If the

component(s) with protease activity on bacitracin mixture can penetrate into the cells, the protease activity may act like calpain to cleave integrin cytoplasmic domain or focal adhesion-related protein, thus interfere with integrin-mediated cell adhesion.

However, the protease activities in bacitracin are sensitive to PMSF. In the presence of PMSF, bacitracin can only cause about 30% of inhibition of $\alpha 5\beta 1$ integrin-mediated cell adhesion. PMSF is a protease inhibitor. It sometimes also inhibits other types of enzymes such as esterases (James GT, 1978). The mechanism of protease inhibition by PMSF is that the sulfonyl floride in PMSF inhibits protease by sulfonylating the hydroxyl group of the serine residue in the active site (Gold AM, 1965). Since bacitracin is a peptide antibiotic, the possibility that such sulfonylation from PMSF on hydroxyl group of the serine residue of the components in bacitracin may also occur and such effects from PMSF may inhibit functions of these components in bacitracin on integrin-mediated cell adhesion.

Since the major components in commercial bacitracin are bacitracin A and bacitracin B, the component(s) with inhibitory effect to integrin-mediated cell adhesion may vary in different batches of bacitracin. This may cause variation of experiment results when different batches or different sources of bacitracin are used. Furthermore, protease activity-free or different fractions of bacitracin are commercially unavailable. All these obstruct further researches in this area. Clearly one could undertake to fractionate bacitracin. However, this was not the focus of this proposal.

2.4.4.2 Other possible effects of bacitracin on integrin-mediated cell adhesion

As mentioned before, some proteases, like Calpain, are very important in the regulation of integrin-mediated cell adhesion. Bacitracin has multiple functions. Bacitracin can inhibit proteases from all four classes: aspartic acid-, serine- and cysteine-proteases, and metallo-exopeptidases (Elbein AD, 1983). Since bacitracin is permeable to mammalian cell membrane, there is a possibility that the protease inhibitory effect of bacitracin may inhibit protease(s) activity in the cytoplasm, therefore, inhibit integrin-mediated cell adhesion.

In addition, bacitracin may also function as an inhibitor of protein glycosylation. Bacitracin has been tested in a number of mammalian systems as an inhibitor of N-linked glycoproteins (Hercovics A et al, 1977; Reuvers F et al, 1978; Chen W and Lennarz W, 1976). When hen oviduct membranes were incubated with UDP-[^{14}C]GlcNAc in the presence of 1 mM bacitracin, a trisaccharide-lipid accumulated. The trisaccharide was characterized as a Man-β-GlcNAc, indicating that bacitracin blocked the addition of the first α-linked mannose residue (Chen W and Lennarz W, 1976). On the other hand, Hercovics A et al found that the formation of dolichyl-pyrophosphoryl-GlcNAc by calf pancreas microsomes was inhibited in the presence of 0.2 to 1 mM bacitracin, but the synthesis of dolichyl-PP-$(GlcNAc)_2$, dolichyl-P-mannose or dolichyl-P-glucose were unaffected (Hercovics A et al, 1977). However, in yeast membrane preparations, 0.33 mM bacitracin was reported to inhibit the formation of dolichyl-PP-$(GlcNAc)_2$, but not dolichyl-PP-GlcNAc (Reuvers F et al, 1978). Therefore, bacitracin is a potent protein N-glycosylation inhibitor. It may act on at least three sites of the glycosylation processes: (1) Bacitracin may form a complex with the lipid carrier, dolichyl-P, thus, inhibiting the formation of lipid-linked monosaccharides and lipid-linked oligosaccharides (Chen W

and Lennarz W, 1976). (2) Bacitracin may also block the transfer of mannose from GDP-mannose to lipid-linked oligosaccharides by the particulate enzyme. (3) Bacitracin can further inhibit the transfer of mannose from dolichyl-P-mannose to lipid-linked oligosaccharides (Spencer J et al, 1978). Since both α and β subunits of the integrins are highly N-glycosylated. Altered glycosylation of β1 integrins have been reported by several papers to be associate with reduced adhesiveness to fibronectin and other ligands. Deglycosylation of α5β1 integrins on K562 cells tended to dissociated α5β1 integrin into α5 and β1 subunits upon immunoprecipitation (Kawano T, 1993; Zheng M, 1994; Kojima N, 1994). These results suggest the important role of N-glycosylation in the integrins functions. Therefore, inhibition of N-glycosylation of the integrins by bacitracin may also contribute to the inhibitory effect of bacitracin to integrin-mediated cell adhesion.

3. Beta-1 integrin conformational change caused by PDI inhibitors tocinoic acid and somatostatin

An interesting discovery in this research is that PDI inhibitors tocinoic acid and somatostatin cause β1 integrin conformational change, leading to exposure of CLIBS. This conformational change is neither accompanied by integrin functional change, nor related to the inhibition of cell surface PDI as inhibitory monoclonal antibody to PDI has no effect on β1 integrin conformational change. One possibility is that this conformational change may be caused by the direct interaction between integrin β1 subunit and tocinoic acid or somatostatin. Both tocinoic acid and somatostatin are synthetic peptides which contain a pair of Cys in their structure (Sigma data sheets).

During the interaction with PDI, this pair of Cys may form mixed disulfide bonds with Cys in PDI, which enhances their nonspecifically binding to peptide-binding site in the C-terminal region and b' domain of PDI (Klappa P et al, 1997; Klappa P et al, 1998). Integrin β1 subunit contains seven C-X-X-C motifs which are considered as a functional motifs for isomerase activity in PDI (Freedman RB, 1994; Erle DJ et al 1991). Therefore, integrin β1 subunit may have protein isomearse activity. Furthermore, integrin β1 subunits have four cystein-rich regions. The C-X-X-C motifs or other cystein(s) in cystein-rich regions in integrin β1 subunit may directly interact with a pair of Cys in tocinoic acid or somatostatin, thus inducing integrin conformational change.

4. Future directions of study

Because of the roles integrins play, regulation of integrin function is important to maintain many normal biological processes. Although the possible role of disulfide exchange mediated by membrane protein disulfide isomerase in the physiological regulation of integrin function is discussed in this research, many questions are still unsolved. First, the present research has demonstrated that conventional membrane PDI on cell surface has no significant effect in the regulation of integrin function in IL-2 dependent peripheral lymphocytes. This does not exclude the possibility that other form(s) of PDI are related to integrin functional regulation. Moreover, the role of PDI-like activity in the integrin-ligand (such as fibronectin) in the regulation of integrin-ligand interaction is unclarified. Recently, integrin β1 and β3 subunits have been identified to have endogenous thiol isomerase activity (O'Neill S et al, 2000). The intrinsic thiol isomerase activity within αIIbβ3 is potent and has high capacitance, equal to that

observed in PDI. The PDI-like activity in the β subunits may be regulated by their individual α subunit in different integrins (O'Neill S et al, 2000). Therefore, the role of PDI-like activity in β subunits in the integrin functional regulation should also be considered. This question can be clarified by testing whether PDI-like activity in integrin β subunits can be inhibited by PDI inhibitors such as DTNB, tocinoic acid and somatostatin, and whether PDI inhibitors have any effects on purified integrins binding to immobilized integrin ligands or soluble integrin ligands binding to purified immobilized integrins. Second, the mechanisms by which bacitracin interferes with integrin-mediated cell adhesion may not be as simple as original suggested. The protease activities in the bacitracin make our research work more difficult since protease activities can interfere with cell adhesion in multiple processes. It can also interfere with soluble ligands binding to purified immobilized integrins. By using protease activity-free bacitracin, the model proposed needs to be further demonstrated. Third, the explanation for the contradictory results relating to the role of membrane PDI in the regulation of integrin function between this research and those from Lahav J et al (Lahav J et al, 2000) requires experimental demonstration. This might be achieved by comparing the effects of PDI inhibitor(s) on integrin-mediated cell adhesion in freshly purified peripheral lymphocytes and IL-2 dependent peripheral lymphocytes. Fourth, whether the integrin β1 subunit conformational changes in the presence of PDI inhibitors are induced by the interaction between PDI inhibitors and membrane PDI or between PDI inhibitors and integrin β subunits are still unknown. Since membrane PDI is not universally expressed on all kinds of cells, the membrane expression of PDI in human skin fibroblasts has not yet been demonstrated (Lahav J et al, 2000). Therefore, by testing the effect of PDI inhibitors on

integrin conformational changes on cells with no detectable membrane PDI, combining with the result obtained by testing the effect of PDI inhibitors on purified integrin binding on immobilized integrin ligands or soluble integrin ligands binding to immobilized purified integrins, this question can also be addressed. Finally, what happens (e.g. signals) inside the cells when PDI inhibitors interact with membrane PDI or directly with integrin is unillustrated. Future studies in this area will still be arduous work.

References

Ahlen K, Rubin K: Platelet-derived growth factor-BB stimulates synthesis of the integrin alpha 2-subunit in human diploid fibroblasts. Exp. Cell Res. 1994, 215: 347.

Akiyama SK, Yamada SS, Yamada KM and Laflamme SE: Transmemvrane signal transduction by integrin cytoplasimc domains expressed in single-subunit chimeras. J. Biol. Chem. 1994, 269: 15961.

Ali IU and Hynes RO: Role of disulfide bonds in the attachment and function of large, external, transformation-sensitive glycoprotein at the cell surface. Biochim. Biophys. Acta. 1977, 510: 140.

Altruda F, Cervella P, Tarone G, Botta C, Balzac F, Stefanuto G, Silengo L: A human integrin β1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. Gene, 1990, 95: 261.

Bacon KB, Premack BA, Gardner P and Schall TJ: Activation of dual T cell signalling pathways by the chemokine RANTES. Science. 1995, 269: 1727.

Balzac F, Belkin AM, Koteliansky VE, Balabanov YV, Altruda F, Silengo L, Tarone G: Expression and functional analysis of a cytoplasmic domain variant of the β1 integrin subunit. J. Cell Biol. 1993, 121: 171.

Bardwell JCA: Building bridges: disulfide bond formation in the cell. Mol. Microbiol. 1994, 14: 199.

Bazzoni G, Shih DT, Buck CA and Hemler ME: Monoclonal antibody 9EG7 defines a novel beta 1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. J. Biol. Chem. 1995, 270: 25570.

Bazzoni G, Ma L, Blue ML and Hemler M: Divalent cations and ligands induce conformational changes that are highly divergent among β1 integrins. J. B. C. 1998, 273: 6670.

Bazzoni G and Hemler ME: Are changes in integrin affinity and conformation overemphasized? TIBS 1998, 23: 30.

Behr S and Schriever F: Engaging CD19 or target of an antiproliferative antibody 1 on human B lymphocytes induces binding of B cells to the interfollicular stroma of human tonsils via integrin alpha4/beta1 and fibronectin. J. Exp. Med. 1995, 182: 1191.

Belkin AM, Zhidkova NI, Balzac F, Altruda F, Tomatis D, Maier A, Tarone G, Koteliansky VE, Burridge K: B1D integrin displaces the β1A isoform in striated muscles: localization at junctional structures and signaling potential in mon-muscle cells. J. Cell Biol. 1996, 132: 211.

Bennett JS, Zigmond S, Vilaire G, Cunningham ME and Bednar B: the platelet cytoskeleton regulates the affinity of the integrin αIIbβ2 for fibrinogen. J. Biol. Chem. 1999, 274(36): 25301.

Berditchevski F, Bazzoni G and Hemler ME: Specific association of CD63 with the VLA-3 and VLA-6 integrins. J. Biol. Chem. 1995, 270 (30): 17784.

Berditchevski F, Zutter MM and Hemler ME: Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol. Biol. Cell 1996, 7: 193.

Bernsten CN, Pettigrew NM, El-Gabalawy HS, Sargent M, Mou YL and Wilkins JA: The differential expression of a novel intestinal epithelial glycoprotein in various forms of inflammatory bowel disease. Am J Clin Pathol. 1996, 106: 42.

Blystone SD, Graham IL, Lindberg FP and Brown EJ: Integrin αvβ3 differentially regulates adhesive and phagocytic functions of the fibronectin receptor α5β1. J. Cell Biol. 1994, 127(4): 1129.

Blystone SD, Lindberg FP, LaFlamme SE and Brown EJ: Integrin β3 cytoplasmic tail is necessary and sufficient for regulation of α5β1 phagocytosis by αvβ3 and integrin-associated protein. J. Cell Biol. 1995, 130(3): 745.

Blystone SD, Slater SE, Williams MP, Crow MT and Brown EJ: A molecular mechanism of integrin crosstalk: αvβ3 suppression of calcium/calmodulin-dependent protein kinase II regulates α5β1 function. J. Cell Biol. 1999, 145: 889.

Bogaert T, Brown N and Wilcox M: The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. Cell. 1987, 51(6): 929.

Brooks P, Stromblad S, Sanders L, von Schalscha T, Aimes R, Stetler-Stevenson W, Quigley J and Cheresh D: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3. Cell. 1996, 85: 683.

Boudreau NJ and Jones PL: Extracellular matrix and integrin signalling: the shape of things to come. Biochem J. 1999, 339: 481.

Brown E, Hooper L, Ho T and Gresham H: Integrin-associated protein: a 50-Kd plasma membrane antigen physically and functionally associated with integrins. J. Cell Biol. 1990. 111: 2785.

Brown MT and Cooper JA: Regulation, substrates and functions of Sre. Biochim. Biophys. Acta. 1996, 1287: 121.

Burn P, Kupfer AC, Singer SJ: Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. Proc. Natl Acad Sci. USA. 1988, 85: 497.

Burridge K, Fath K, Keily T, Nuckolls G, Turner C: Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. 1988, 4: 487.

Caixia S, Stewart S, Wayner EA, Carter WG and Wilkins JA: Antibodies to different members of the β1 integrins induce homotypic and heterotypic cellular aggregation. Cell. Immunol. 1991, 138: 218.

Calvete JJ, Henschen A, Gonzalez-Rodriguez J: Assignment of disulfate bonds in human platelet GPIIIa: A disulfate pattern for the beta-subunit of the integrin family. Biochem J. 1991, 274: 63.

Calvete JJ: Clues for understanding the structure and function of a prototypic human integrin: the platelet glycoprotein IIb/IIIa complex. Thrombosis and Haemostasis. 1994, 72(1): 1.

Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA and Butcher EC: Chemokines and the arrest of lymphocytes rolling under flow conditions. Science. 1998, 279(16): 381.

Cao Z, Huan K and Horwitz AF: Identification of a domain on the integrin α5 subunit implicated in cell spreading and signaling. J. Biol. Chem. 1998, 273: 31670.

Carlos TM and Harlan JM: Leukocyte-endothelial adhesion molecules. Blood 1994, 84(7): 2068.

Carpen O, Pallai P, Staunton DE, Springer TA: Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and α actinin. J. Cell Biol. 1992, 118: 1223.

Chan BM, Wong JG, Rao A and Hemler ME: T cell receptor-dependent, antigen-specific stimulation of a murine T cell clone induces a transient, VLA protein-mediated binding to extracellular matrix. J. Immunology, 1991, 147: 398.

Chan BM, Kassner PD, Schiro JA, Byers HR, Kupper TS and Hemler ME: Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell 1992, 68: 1051.

Chang DD, Wong C, Smith H and Liu J: ICAP-1, a novel β1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of β1 integrin. J. Cell Biol. 1997, 138(5): 1149.

Chen K, Lin Y and Detwiler TC: Protein disulfide isomerase activity is released by activated platelets. Blood, 1992, 79(9): 2226.

Chen WW and Lennarz WJ: Participation of a trisaccharide-lipid in glycosylation of oviduct membrane glycoproteins. J. Biol. Chem. 1976, 251: 7802.

Chicurel ME, Singer RH, Meyer CJ and Ingber DE: Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. Nature 1998, 392, 730.

Chothia C and Jones E: The molecular structure of cell adhesion molecules. Annaul Rev. Biochem. 1997, 66: 823.

Clark EA and Brugge JS: Integrins and signal transduction pathways: the road taken. Science 1995, 268: 233.

Cohen GB, Ren R, Baltimore D: Modular binding domsins in signal transduction proteins. Cell. 1995, 80(2): 237.

Collo G, Starr L and Quaranta V: A new isoform of the laminin receptor integrin alpha 7 beta 1 is developmentally regulated in skeletal muscle. J. Biol. Chem. 1993, 268 (25): 19019.

Cooper DT, Chiu H, Fong S and Weissman IL: The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. J. Cell Biol. 1991, 115(3): 843.

Cooray P, Yuan U, Schoenwaelder SM, Mitchell CA, Salem HH and Jackson SP: Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. Biochem. J. 1996, 318: 41.

Couet J, Bernard S, Loosfelt H, Saunier B, Milgrom E and Misrahi M: Cell surface protein disulfide-isomerase is involved in the shedding of human thyrotropin receptor ectodomain. Biochem. 1996, 35: 14800.

Critchley DR, Holt MR, Barry ST, Priddle H, Hemmings L and Norman J: Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem. Soc, Symp. 1999, 65: 79.

Crowe DT, Chiu H, Fong S and Weissman IL: Regulation of the avidity of integrin alpha 4 beta 7 by the beta 7 cytoplasmic domain. J. Biol. Chem. 1994, 269 (20): 14411.

Dana N, Fathallah DM and Arnaout MA: Expression of a soluble and functional form of the human beta2 integrin CD11b/CD18. Pro. Natl. Acad. Sci. U. S. A. 1991, 88(8): 3106.

Davis GE and Camarillo CW: Regulation of integrin-mediated myeloid cell adhesion to fibronectin: influence of disulfide reduing agents, divalent cations and phorbol ester. J. Immunol. 1993, 151(12): 7138.

Defilippi P, van Hinsbergh V, Bertolotto A, Rossino P, Silengo L and Tarone G: Differential distribution and modulation of expression of alpha 1/ beta 1 integrin on human endothelial cells. J Cell Biol. 1991, 114: 855.

Deryugina E, Bourdon M Lou GX, Reisfeld R and Strongin A: Matrix metalloproteinase activation modulates glioma cell migration. J. Cell Sci. 1997, 110: 2473.

DeSimon DW and Hynes RO: Xenopus laevis integrins. Structural conservation and evolutionarydivergence of integrin beta subunits. J. Biol. Chem. 1988, 263(11): 53333.

Diamond MS and Springer TA: A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J. Cell Biol. 1993, 120: 545.

Diamond MS, Springer TA: The dynamic regulation of integrin adhesiveness. Curr Biol. 1994, 4: 506.

Diamond MS, Staunton DE, Marlin SD, Springer TA: Binding of the integrin Mac-1 (CD11b/Cd18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell. 1991, 65: 961.

Dickeson SK and Santoro SA: Ligand recognition by the I domain-containing integrins. CMLS, Cell. Mol. Life Sci. 1998, 54: 556.

Dogic D, Rousselle P and Aumailley M: Cell adhesion to laminin 1 or 5 induces isoform-specific clustering of integrins and other focal adhesion components. J. Cell Sci. 1998, 111: 793.

D'Souza SF, Ginsberg MH, Burke TA, Lam SC and Plow EF: Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. Science. 1988, 242 (4875): 91.

D'Souza SF, Ginsberg MH, Burke TA and Plow EF: The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its alpha subunit. J. Biol. Chem. 1990, 265 (6): 3440.

D'Souza SF, Ginsberg MH, Matsueda GR and Plow EF: A discrete sequence in a platelet integrin is involved in ligand recognition. Nature. 1991, 350 (6313): 66.

Dransfield I and Hogg N: Regulation expression of Mg++ binding epitope on leukocyte integrin α subunits. EMBO. 1989, 8: 3759.

Du X, Plow E, Frelinger AL, O'Toole T, Loftus J and Ginsberg M: Ligands "activate" integrin αIIbβ3 (Platelet GPIIb-IIIa). Cell, 1991, 65: 409.

Du X, Saido, TC, Tsubuki S, Indig FE, Williams MJ and Ginsberg MH: Calpain cleavage of the cytoplasmic domain of the integrin beta3 subunit. J. Biol. Chem. 1996, 270, 26246.

Dunon D, Mackay CR and Imhof BA: Adhesion in leukocyte homing and differentiation. 1993, PP260. Springer Verlag, New York.

Edwards BS, Curry MS, Southon EA, Chong ASF and Graf LH: Evidence for a dithiol-activated signaling pathway in matural killer cell avidity regulation of leukocyte function antigen-1: structural requirments and relationship to phorbol ester-and CD16-triggered pathways. Blood, 1995, 86: 2288.

Elbein AD: Inhibitors of the biosynthesis and processing of N-linked oligosaccharides. CRC Crit. Rev. Biochem. 1983, 16: 21.

El-Gabalawy HS, King R, Bernstein C. Ma GP, Mou YL, Alguacil-Garcia A, Fritzler M and Wilkins JA: Expression of N-acetyl-D-galactosamine associated epitope in synovium: a potential marker of glycoprotein production. J. Rheumatol. 1997, 24: 1355.

Erle DJ, Ruegg C, Sheppard D and Pytela R: Complete amino acid sequence of an integrin β subunit (β7) identified in leukocytes. J. Biol. Chem. 1991, 266: 11099.

Essex DW, Chen K and Swiatkowska M: Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. Blood 1995, 86(6): 2168.

Essex DW and Li M: Protein disulphide isomerase mediates platelet aggregation and secretion. British J. Haematology, 1999. 104: 448.

Fassler R and Meyer M: Consequences of lack of β1 integrin gene expression in mice. Gene Dev. 1995, 9: 1896.

Faull RJ: Dynamic regulation of integrins. Stem Cells. 1995, 13: 38.

Feener EP, Shen WC and Ryser HJP: Cleavage of disulfide bonds in endocytosed macromolecules. A processing not associated with lysosomes or endosomes. J. Biol. Chem. 1990, 265: 18780.

Filardo EJ, Brooks PC, Deming SL, Damsky C and Cheresh DA: Requirement of the NPXY motif in the integrin β3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. J. Cell Biol. 1995, 130: 441.

Freedman RB, Hirst TR and Tuite MF: Protein disulphide isomerase: building bridges in protein folding. Trends. Biochem. Sci. 1994, 19: 331.

Fujii K, Dousaka-Nakajima N, Imamura S: Epidermal growth factor enhancement of HSC-1 human cutaneous squamous carcinoma cell adhesion and migration on type I collage involves selective up-regulation of alpha 2 beta 1 integrin expression. Exp Cell Res. 1995, 216: 261.

Fukai GF, Ohataki M, Yajima H, Ishii T, Nishizawa Y, Migazaki K and Katayama T: Release of biological activities from quiescent fibronectin by a conformational change and limited proteolysis by matrix metalloproteinases. Biochemistry. 1995, 34: 11453.

Gao JX, Wilkins JX Issekutz AC: Migration of human polymorphonuclear leukocytes through a synovial fibroblast barrier is mediated by both β2 (CD11/CD18) integrin and the β1 (CD29) integrins. Cell. Immunol. 1995, 163: 178.

George E: Regulation of integrin-mediated myeloid cell adhesion to fibronectin: influence of disulfied reducing agents, divalent cations and phorbol ester. J. Immunol. 1993, 151:7138.

Germain RH and Marguiles SM: The biochemistry and cell biology of antigen processing and presentation. Ann Rev. Immunol. 1993, 11: 403.

Ginsberg MH, Du X and Plow EF: Inside-out integrin dignalling. Curr. Opin Cell Biol. 1992, 4: 766.

Ginsberg MH: IntegrinsL dynamic regulation of ligand binding. Biochem Soc Trans. 1995, 23: 439.

Gofer-Dadosh N, Klepfish A, Schmilowitz H, Shaklai M and Lahav J: Affinity modulation in platelet alpha2 beta 1 following ligand binding. Biochem. Biophys. Res. Commun. 1997, 232(3): 724.

Gold AM: Sulfonyl fluorides as inhibitors of esterases. III. Identification of Serine as the site of sulfonylation in Phenylmethanesulfonyl α-Chymotrypsin. Biochemistry. 1965, 4(5): 897.

Goldman H, Scriver CR, Aaron K and Pinsky L: Use of dithiothreitol to correct cystine storage in cultured cystinotic fibroblasts. Lancet, 1970, 18: 811.

Gresham HD, Goodwin JL, Anderson DC and Brown EJ: A novel member of integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. J. Cell Biol. 1989, 108: 1935.

Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y and Tedder TF: Granulocyte macrophage colony stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors. J. Immunol. 1990, 145: 576.

Grimaldi CM, Chen F, Scudder LE, Coller BS and French DL: A Cys374Tyr homozygous mutation of platelet glycoprotein IIIa (beta 3) in a Chinese patient with Glanzmann's thrombasthenia. Blood. 1996, 88(5): 1666.

Guinebault C, Payrastre B, Racaud-Sultan C, Mazarguil H, Breton M, Mauco G, Plantavid M and Chap H. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytokeleton of thrombin-activated platelets involves specific interactions of P85 alpha with actinfilaments and focal adhesion kinase. J. Cell Biol. 1995, 129: 831.

Hanks SK and Polte TR: Signaling through focal adhesion kinase. BioEssays 1997, 19(2): 137.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J. Bell JC and Dedhar S: Regulation of cell adhesion and anchorage-dependent growth by a new beta1 integrin-linked protein kinase. Nature 1996, 379: 91.

Harlan JM: Leucocyte adhesion deficiency syndrome: insights into the molecular basis of leucocyte emigration. Clin Immunol. Immunopathol. 1993, 67: S16.

Heiska L, Kantor C, Parr T, Critchley DR, Vilja P, Gahmberg CG and Carpen O: Binding of the cytoplasmic domain of intercellular adhesion molecule 2 (ICAM-2) to a-actinin. J. Biol. Chem. 1996, 271: 26214.

Heiska L, Alfthan K, Gronholm M, Vilja P, Vaheri A and Carpen O: Association of ezrin with intercellular adhesion molecule-1 and –2 (ICAM-1 and ICAM-2). J. Bio. Chem. 1998, 273: 21893.

Helander TS, Carpen O, Turunen O, Kovanen PE, Vaheri A and Timonen T: ICAM-2 redistributed by ezrin as a target for killer cells. Nature. 1996, 382: 265.

Heldin CH: Dimerization of cell surface receptors in signal transduction. Cell. 1995, 80 (2): 213.

Hemler ME: VLA proteins in the integrin family: structure, functions, and their role on leukocytes. Annual Review of Immunology. 1990, 8: 365.

Hemler ME, Kassner PD, Chan BMC: Functional roles for integrin α subunit cytoplasmic domains. Cold spring harbor symposia on quantitative biology. 1992, Vol VVII: 213.

Hercovics A, bugge B and Jeanloz RW: Effect of bacitracin on the biosynthesis of dolichol derivatives in calf pancreas microsomes. FEBS. Lett, 1997, 82: 215.

Hibbs ML, Jakes S, Stacker SA, Wallace RW and Springer TA: The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 beta subunit: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. J. Exp. Med. 1991, 174: 1227.

Hildebrand JD, Schaller MD, Parsons JT: Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125 FAK, to cellular focal adhesions. J. Cell Biol. 1993, 123: 993.

Hillis GS and Macleod AM: Integrins and disease. Clinical Science. 1996, 91: 639.

Hirsch E, Iglesias A, Potocnik AJ, Hartmann V, Fassier R: Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta 1 integrins. Nature. 1996, 380(6570): 171.

Hocking DC, Smith RK and McKeown-Longo PJ: A novel role for the integrin-binding III-10 module in fibronectin matrix assembly. J. Cell Biol. 1996, 133: 431.

Hocking DC, Sottile J and McKeown-Longo PJ: Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. J. Biol. Chem. 1994, 269: 19183.

Honda S, Tomiyama Y, Pelletier AJ, annis d, Hond Y, Orchekowski R, Ruggeri Z and Kunick TJ: topography of ligand-induced binding sites, including a novel cation-senitive epitope (AP5) at the amino terminus, of the human integrin β3 subunit. J. Biol. Chem. 1995, 270(20): 11947.

Horton MA: Molecular biology of cell adhesion molecules. 1996.

Horvath G , Serru V, Clay D, Billard M, Boucheix C and Rubinstein E: CD19 is linked to the integrin-associated Tetraspans CD9, CD81 and CD82. J. Biol. Chem. 1998, 273(46): 30537.

Hugues JPR, Levy EM, Mandel R and DiSciullo GJ: Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. Proc. Natl. Acad. Sci. USA 1994, 91: 4559.

Hughes PE, O'Toole TE, Ylanne J, Shattil SJ and Ginsberg MH: The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. J. Biol. Chem. 1995, 270: 12411.

Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ and Ginsberg MH: Breaking the integrin hinge: a defined structural constraint regulates integrin signaling. J. Biol. Chem. 1996, 271: 6571.

Huttenlocher A, Palecek SP, Lu Q, Zhang WL, Mellgren RL, Lauffenburger DA, Ginsberg MH and Horwitz AF: Regulation of cell migration by the calcium-dependent protease Calpain. J. Biol. Chem. 1997, 272: 32719.

Hynes RO: Integrin: versatility, modulation, and signaling in cell adhesion. Cell. 1992, 69: 11.

Ignotz RA and Massague J: Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell. 1987, 51: 189.

Imhof BA And Dunon D: Leukocyte migration and adhesion. Adv. Immunol. 1995, 58: 345.

Inomata M, Hayashi M, Ohno-Iwashita Y, Tsubuki S, Saido TC and Kawashima S: Involvement of calpain in integrin-mediated signal transduction. Arch. Biochem. Biophys. 1996, 328: 129.

Irie A, Kamata T, Puzon-McLaughlin W and Takada Y: Critical amino acid residues for ligand binding are clustered in a predicted beta-turn of the third N-terminal repeat in the integrin alpha 4 and alpha 5 subunits. EMBO J. 1995, 14: 5550.

Jakubowski A, Rosa MD, Bixler S, Lobb R and Burkly LC: Vascular cell adhesion molecule (VCAM)-Ig fusion protein defines distinct affinity states of the very late antigen-4 (VLA-4) receptor. Cell adhes. Commun. 1995, 3: 131.

James GT: Inactivation of the protease inhibitor Phenylmethylsulfonyl Fluoride in brffers. Analytical Biochemistry. 1978, 86: 574.

Janat MF, Argarves WS, Liau G: Regulation of vascular smooth muscle cell integrin expression by transforming growth factor beta 1 and by pplatelet-derived growth factor-BB. J. Cell Physiol. 1992, 151: 588.

Jones J, Sugiyama M, Speight PM and Watt FM: Restoration of αvβ5 integrin expression in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage-independent growth. Oncogene. 1996, 12: 119.

Jones SL, Wang J, Turck CW and Brown EJ: A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function. Proc. Natl. Acad. Sci. USA 1998, 95: 9331.

Kaetzel CS, Rao CK and Lamm ME: Protein disulfide-isomerase from human placenta and rat liver. Purification and immunological characterization with monoclonal antibodies. Biochem. J. 1987, 241: 39.

Kamata T and Takada Y: Direct binding of collagen to the I domain of integrin alpha 2 beta 1 (VLA-2, CD49b/CD29) in a divalent cation-independent manner. J. Biol. Chem. 1994, 269 (42): 26006.

Kamata T, Puzon W and Takada Y: Identification of putative ligand binding sites within I-domain of integrin α2β1 (VLA-2, CD49b/CD29). J. Biol. Chem. 1994, 269: 9659.

Kamata T, Wright R and Takada Y: Critical Thr and Asp residues within the I-domains of β2 integrins for interactions with ICAM-1 and C3bi. J. Biol. Chem. 1995, 270: 12531.

Kanner SB, Grosmaire LS, Ledbetter JA and Damle NK: Beta 2 integrin LFA-1 signaling through phospholipase C-gamma 1 activation. Proc. Natl. Acad. Sci. USA. 1993, 90 (15): 7099.

Kassner PD and Hemler ME: Interchangeable α chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a β1 integrin. J. Exp. Med. 1993, 174: 1227.

Kawaguchi S and Hemler ME: Role of the α subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. J. Biol. Chem. 1993, 268: 16279.

Kawano T, Takasaki S, Tao TW, Kobata A: Altered glycosylation of beta 1 integrins associated with reduced adhesiveness to fibronectin and laminin. Int. J Cancer. 1993, 53: 91.

Kawasaki H and Kretsinger RH: Calcium-binding proteins 1: EF hands. Protein-Profile. 1995, 2 (4): 297.

Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, Inoue S, Yoshino K, Okumura K and Yagita H: Metalloproteinase-mediated release of human Fas ligand. J. Exp. Med. 1996, 182: 1777.

Kern A Briesewitz R, Bank I and Marcantonio E: The role of the I-domain in ligand binding of the human integrin $\alpha 1\beta 1$. J. Biol. Chem. 1994, 269: 22811.

Kirchberg K, Lange TS, Klein EC, Jungtaubl H, Heinen G, Meyer-Ingold W, Scharffetter-Kochanek K: Inductin of beta 1 integrin synthesis by recombinant platelet-derived growth factor (PDGF-AB) correlates with an enhanced migratory response of human dermal fibroblasts to various extracellular matrix proteins. Exp Cell Res. 1995, 220: 29.

Khan K and Falcone D: Role of laminin in matrix induction of macrophage urokinase-type plasminogen activator and 92-kDa metalloproteinase expression. J. Bio. Chem. 1997, 272: 8270.

Klappa P, Hawkins HC and Freedman RB: Interactions between protein disulphide isomerase and peptides. Eur. J. Biochem. 1997, 248: 37.

Klappa P, Ruddock LW, Darby NJ and Freedman RB: The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. EMBO 1998, 17: 927.

Kobayashi H, Ohi H, Sugimura M, Shinohara H, Fujii T and Terao T: Inhibition of in vitro ovarian cancer cell invasion by modulation of urokinase-type plasminogen activator and cathepsin B. Cancer Res. 1992, 52: 3610.

Kojima N, Saito M, Tsuji S: Role of cell surface O-linked oligosaccharides in adhesion of HL60 cells to fibronectin: regulation of integrin-dependent cell adhesion by O-linked oligosaccharide elongation. Exp. Cell Research. 1994, 214: 537.

Kouns, WC, Steiner B, Kunicki TJ, Moog S, Jutzi J, Jennings LK, Cazenave JP and Lanza F: Activation of the fibrinogen binding site on platelets isolated from a patient with the Strasbourg I variant of Glanzmann's thrombasthenia. Blood, 1994, 1108.

Kraal G. and Mebius R. E: High endothelial venules: lymphocyte traffic control and controlled traffic. Adv. Immunol. 1997, 65: 347.

Kroning H, Kahne T, Ittenson A, Franke A, Ansorge S: Thiol-proteindisulfide-oxidoreductase (proteindisulfide isomerase): A new plasma membraneconstituent of mature human B lymphocytes. Scand J. Immunol. 1994, 39: 346.

Kruisbeek AM and Amsen D: Mechanisms underlying T-cell tolerance. Cur Opin. Immunol. 1996, 8: 233.

Kumar CC: Signaling by integrin receptors. Oncogene. 1998, 17: 1365.

Lahav J, Gofer-Dadosh N, Luboshitz J, Hess O and Shaklai M: Protein disulfide isomerase mediates integrin-dependent adhesion. FEBS Letters. 2000, 475: 89.

Landis R, McDowall A, Holness C, Littler A, Simmons D and Hogg N: Involvement of the "I" domain of LFA-1 in selective binding to ligands ICAM-1 and ICAM-3. J. Cell Biol. 1994, 126(2): 529.

Langenbach KJ and Sottile J: Identification of protein-disulfide isomerase activity in fibronectin. J. Biol. Chem. 1999, 274: 7032.

Languino LR and Ruoslahti E: An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. J. Biol. Chem. 1992, 267: 7116.

Lee OJ, Rieu P, Arnaout MA and Liddington R: Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). Cell 1995, 80: 631.

Lenter M, Uhlig H, Hammann A, Jeno P, Imhof B and Vestweber D: A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha6 beta1. Proc. Natl. Acad. Sci. USA, 1993, 90: 9051.

Leventhal PS and Feldman EL: Tyrosine phosphorylation and enhanced expression of paxillin during neuronal differentiation in vitro. J. Biol. Chem. 1996, 271 (11): 5957.

Ley K and Tedder TF: Leukocyte interactions with vascular endothelium. New insights into selectin-mediated attachment and rolling. J. Immunol. 1995, 155: 525.

Li R, Nortamo P, Kantor C, Kovanen P, Timonen T and Gahmberg C: A leukocyte integrin binding peptide from intercellular adhesion molecule-2 stimulates T cell adhesion and natural killer cell activity. J. Biol. Chem. 1993, 268: 21474.

Li R, Xie J, Kantor C, Koistinen V, Altieri D, Nortamo P and Gahmberg C: A peptide derived from the intercellular adhesion molecule-2 regulates the avidity of the leukocyte integrins CD11b/CD18 and CD11c/CD18. J. Cell Biol. 1995, 129, 1143.

Lin EC, Ratnikov BI, Tsai PM, Carron CP, Myers DM, Barbas CF and Smith JW: Identificationof a region in the integrin β3 subunit that confers ligand binding specificity. J. Biol. Chem. 1997, 272: 23912.

Lindberg FP, Gresham HD, Schwarz E and Brown EJ: Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alphav beta3-dependent ligand binding. J. Cell Biol. 1993, 123: 485.

Liu CY, Sun QH, Wang R, Paddock CM and Newman PJ: Further examination of the role of GPIIIa long range Cys5-Cys435 disulfide bond in conformational changes of GPIIb-IIIa: Substitution of cysteine 5 with alanine results in the production of a constitutively active GPIIb-IIIa integrin complex. Blood, 1998, 92: A1413.

Loftus JC, D'Toole TE, Plow EF, Glass A, Frelinger AL 3d and Ginsberg MH: A beta 3 integrin mutation abolishes ligand binding and alters divalent action-dependent conformation. Science. 1990, 249 (4971): 915.

Lollo BA, Chan KWH, Hanson EM, Moy VT and Brian AA: direct evidence for two affinity states for lymphocyte fucntion-associated antigen 1 on activated T cells. J. Biol. Chem. 1993, 268: 21693.

Lub M, van Kooyk Y, van Vliet SJ and Figdor CG: Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. Mole. Biol. Cell 1997, 8: 341.

Lukashev ME, Sheppard D and Pytela R: Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed β1 integrin cytoplasmic domain. J. Biol. Chem. 1994, 269: 18311.

Luque A, Gomez M, Puzon W, Takada Y, Sanchez-Madrid F and Cabanas C: Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355-425) of the common β1 chain. J. Biol. Chem. 1996, 271: 11067.

Luz JM and Lennarz WJ: Protein disulfide isomerase: a multifunctional protein of the endoplasmic reticulum. EXS. 1996, 77: 97.

Lunam EB, Rogelj S, Edwards BS and Sklar LA:Enhanced aggregation of human neutrophils by MnCl2 or DTT differentiates the roles of L-selectin and beta 2-integrins. J. Leukoc. Biol. 1996, 60(3): 356.

Mackay, CR: Immunological memory. Advances in immunology, 1993, 53: 217.

Maecker H, Todd SC and Levy S: The tetraspanin superfamily: molecular facilitators. FASEB J, 1997, 11: 428.

Makinen KK: Inhibition by bacitracin of some hydrolytic enzymes. Inter J Pro. Research. 1972, 4: 21.

Mandel R, Hugues JPR, Ghani F, Wu M and Peak D: Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. Proc. Natl. Acad. Sci. USA 1993, 90: 4112.

Markovic Z, Lustig A, Engel J and Hormann H: Shape and stability of fibronectin in solutions of different pH and ionic strength. Hoppe-Seyler's Z. Phys. Chem. 1983, 364: 1795.

Masumoto A and Hemler ME: Mutation of putative divalent cation sites in the alpha 4 subunit of the integrin VLA 4, distinct effects on adhesion to CD1/fibronectin, VCAM-1, and invasin. J. Cell Biol. 1993, 123: 245.

McGeehan G, Becherer J, Bast R, Boyer C, Champion B, Connolly K, Furdon P, Karp S and Kidao S: Regulation of tumor necrosis factor-alpha processing by a metalloproteinase inhibitor. Nature 1994, 370: 558.

McKeown-Longo PJ and Mosher DF: Binding of plasma fibronectin to cell layers of human skin fibroblasts. J. Cell Biol. 1983, 97: 466.

Medina V, Kesner L and Strasher A: Purification of nonantibiotic insulinase inhibitors from bacitracin. Biochem. Med, and Metabol. Biol. 1993, 49: 255.

Meredith J, Takada Y, Fornaro M, Languino LR and Schwartz MA: Inhibition of cell cycle progression by the alternatively spliced integrin β1C. Science 1995, 268: 1579.

Michishita M, Videm V and Arnaout MA: A novel divalent cation-binding site in A domain of the beta 2 integrin CR3 (CD11b/CD18) is essential for lignad binding . Cell. 1993, 72 (6): 857.

Miller LJ, Bainton DJ, Borregard N, Springer TA: Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J. Clin Invest. 1987, 80: 535.

Mizunaga T, Katakura Y, Miura T and Maruyama Y: Purification and characterization of yeast protein disulfide isomerase. J. Biochem. 1990, 108: 846.

Molad Y, Haines KA, Anderson DC, Buyon JP, Cronstein BN: Immunocomplexes stimulate different signalling events to chemoattractants in the neutrophil and regulate L-selectin and beta 2 integrin expression differently. Biochem J. 1994, 299: 881.

Molineaux S, Casano F, Rolando A, Peterson E, Limjuco G, Chin J, Griffin P, Calaycay J, Ding G and Yamin T: Interleukin 1 beta (IL-1 beta) processing in murine macrophages requires a structurally

conserved homologue of human IL-1 beta converting enzyme. Proc. Natl. Acad. Sci. U. S. A. 1994, 90: 1809.

Monier-Gavelle F and Duband JL: Cross talk between adhesion molecules: control of N-cadherin activity by intracellular signals elicited by beta 1 and beta 3 integrin in migrating neural crest cells. J. Cell Biol. 1997, 137: 1663.

Morjana NA and Gilbert HF: Effect of protein and peptide inhibitors on the activity of protein disulfide isomerase. Biochemistry 1991, 30: 4985.

Morla A and Ruoslahti E: A fibronectin self-assembly site involved in fibronectin matrix assembly: reconstruction in synthetic peptide. J. Cell. Biol. 1992, 118(2): 421.

Mou YL, Ni HY, Wilkins JA: The selective inhibition of β1 and β7 integrin-mediated lymphocyte adhesion by bacitracin. J. Immunol. 1998, 161: 6323.

Mould AP: Getting integrins into shape: recent insights into how integrin activity is regulated by conformational changes. J. Cell Sci. 1996a, 109: 2613.

Mould AP, Akiyama SK and Humphries MJ: The inhibitory anti-beta 1 integrin monoclonal antibody 13 recognizes an epitope that is attenuated by ligand occupancy. Evidence for allosteric inhibition. J. Biol. Chem. 1996b, 271: 20365.

Mould AP, Akiyama SK and Humphries MJ: Regulation of integrin α5β1-fibronectin interaction by divalent cations. J. Bio. Chem. 1995a, 270: 26270.

Mould AP , Garratt AN, Askari JA and Humphries MJ: Identification of a novel anti-integrin monoclonal antibody that recognises a ligand-induced binding site epitope on the beta 1 subunit. FEBS Lett. 1995b, 363: 118.

Mullberg J, Durie F, Otten-Evans C Alderson M, Rose-John s, Cosman D, Black R and Mohler K: A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. J. Immunol. 1995, 155: 5198.

Muller WA, Weigl SA, Denf X and Philips DM: PECAM-1 is required for transendothelial migration of leukocytes. J. Exp. Med. 1993, 178: 449.

Munoz M, Serrador J, Sanchez-Madrid F and Teixido J: A region of the integrin VLAα4 subunit involved in homotypic cell aggregation and in fibronectin but not vascular cell adhesion molecule-1 binding. J. Biol. Chem. 1996, 271: 2696.

Nakamura K, Iwamoto R and Mekada E: Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a xomplex with integrin alpha 3 beta 1 at cell-cell contact sites. J. Cell Biol. 1995, 129: 1691.

Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F and Comoglio P: Biological activation of pro-HGF (hepatocyte to growth factor) by urokinase is controlled by a stoichiometric protein. J. Biol. Chem. 1995, 270: 903.

Neelamegham S, Simon SI, McIntyre BW and Zygourakis K: Induction of homotypic lymphocyte aggregation: evidence for a novel activation state of the β1 integrin. J. Leuk. Biol. 1996, 59: 872.

Ng-Sikorski J, Andersson R, Patarroyo M and Andersson T: Calcium signaling capacity of the CD11b/CD18 integrin on human neutrophils. Exp. Cell. Res. 1991, 195 (2): 504.

Ni HY, Wilkins JA: Localization of a novel adhesion blocking epitope on the human β1 chain, Cell adhesion and Communication. 1998a, 5: 257)

Ni HY, Li AL, Simonsen N, Wilkins JA: Integrin activation by dithiothreitol or Mn++ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta 1 integrin chain. J. Biol. Chem. 1998b, 273: 7981.

Ni HY, Mou YL, McGlaughlin-Puzon W, Takada Y and Wilkins JA: "Evidence for long range and intracellular regulation of the exposure of a cation and ligand induced binding site (CLIBS) on beta-1 integrin". Submitted to Journal of Immunology.

Ni J, Porter AG and Hollander D: Beta 7 integrins and other cell adhesion molecules are differentially expressed and modulated by TNF beta in different lymphocyte populations. Cell Immunol. 1995, 161: 166.

Niiya K, Hodson E, Bader R, Byers-Ward V, Koziol JA, Plow EF, Ruggeri ZM: Increased surface expression of the membrane glycoprotein Iib/IIIa complex induced by platelet activation. Relationship to the binding of fibrinogen and platelet aggregation. Blood 1987, 70: 475.

Novia R, Kimura H, Roos J and Lennarq WJ: Peptide binding by protein disulfide isomerase, a resident protein of the endoplasmic reticulum lumen. J. Biol. Chem. 1991, 166: 19645.

Noiva R and Lennarz WJ: Protein disulfide isomerase-a multifunctinal protein resident in the lumen of the endoplasmic reticulum. H. Biol. Chem. 1992, 267(6): 3553.

Noiva R, Freedman, RB and Lennarz WJ: Peptide binding to protein disulfide isomerase occurs at a site distinct from the active site. J. Biol. Chem. 1993, 268: 19210.

O'Neill S, Robinson A, Deering A, Ryan M, Fitzgerald DF and Moran N: The platelet integrin αIIbβ3 has an endogenous thiol isomerase activity. J. Biol. Chem. 2000, Aug. 14: in press.

Ortlepp S, Stephens PE, Hogg N, Figdor CG and Robinson MK: Antibodies that activate beta 2 integrins can generate different ligand binding states. Eur. J. Immunol. 1995, 25 (3): 637.

O'Toole TE, Ylanne J and Culley BM: Regulation of integrin affinity states through an NPXY motif in the subunit cytoplasmic domain. J. Biol. Chem. 1995, 270: 8553.

O'Toole TE: Integrin signaling: Building connections beyond the focal contact? Matrix Biol. 1997, 16: 165.

Ozaki Y, Satoh K, Kuroda K, Qi R, Yatomi Y, Yanagi S, Sada K, Yamamura H, Yanabu M, Nomura S: Anti-CD9 monoclonal antibody activates p72syk in human platelets. J. Biol. Chem. 1995, 270: 15119.

Pacifici R, Roman J, Kimble R, Civitelli R, Brownfield CM and Bizzarri C: Ligand binding to moncyte α5β1 integrin activates the α2β1 receptor via the α5 cytoplasmic domain and protein kinase C. J. Immunol. 1994, 153: 2222.

Panetti TS and Mckeown-Longo PJ: The αvβ5 integrin receptor regulates receptor-mediated endocytosis of vitronectin. 1993, 268(16): 11492.

Pankonin G, Reipert B and Ager A: Interaction between IL-2-activated lymphocytes and vascular endothelium: binding to and migration across specialized and non-specialized endothelia. Immunology. 1992, 77(1): 51.

Parise LV, Criss AB Nannizzi L and Wardell MR: Glycoprotein IIIa is phosphorylated in intact human platelets. Blood, 1990, 75: 2363.

Parsons JT: Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. Curr. Opin. Cell Biol. 1996, 8 (2): 146.

Pasqualini R, Bodorova J, Ye S and Hemler ME: A study of the structure, function and distribution of β5 integrins using novel anti-β5 monoclonal antibodies. J. Cell Sci. 1993, 105: 101.

Pawson R, A'Hern R and Catovsky D: Second malignancy in hairy cell leukaemia: no evidence of increased incidence agter treatment with interferon alpha. Leuk. Lymphoma. 1996, 22 (1-2): 103.

Paydas S, Yuregir GT, Sahin B, Seyrek E, Burgut R: Intracellular glutathione content in leukemias. Oncology, 1995, 52: 112.

Peerschke EI: Regulation of platelet aggregation by post-fibrinogen binding events. Insights provided by dithiothreitol-treated platelets. Thromb. Haemostasis, 1995, 73: 862.

Pensa B, Costa M, Pecci L, Cannella C and Cavallini D: Selective reactivity of rhodanese sulfhydryl groups with 5,5'-dithio-bis (2-nitrobenzoic acid). Biochimica et Biophysica acta. 1977, 484: 368.

Peter K and O'Toole TE: Modulation of cell adhesion by changes in alpha L beta 2 (LFA-1, CD11a/CD18) cytoplasmic domain/cytoskeleton interaction. J. Exp. Med. 1995, 181: 315.

Pfaff M, Du X and Ginsberg M: Calpain cleavage of integrin β cytoplasmic domains. FEBS, 1999, 460: 17.

Pigiet VP and Schuster BJ: Thioredoxin-catalyzed refolding of disulfide-containing proteins. Proc. Natl. Acad. Sci. USA 1986, 83: 7643.

Plopper G, McNamee HP, Dike E, Bojanowski K and Ingber DE: Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol. Biol. Cell. 1995, 6(10): 1349.

Polte TR and Hanks SK: Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130 cas. Proc. Natl. Acad. Sci. USA. 1995, 92: 10678.

Porter JC and Hogg N: Integrin cross talk: activation of lymphocyte function-associated antigen-1 on human T cells afters alpha 4 beta 1 and alpha 5 beta 1-mediated function. J. Cell Biol. 1997 138: 1437

Postigo AA, Sanchez-Mateos P, Lazarovits AI, Sanchez-Madrid F and de Landazuri MO: α4β7 integrin mediates B cell binding to fibronectin and vascular cell adhesion molecule-1. J. Immunol. 1993, 151: 2471.

Qu A and Leahy DJ: Crystal structure of the I-domain from the CD11a/CD18 (LFA-1,alpha L beta 2) integrin. Proc. Natl. Acad. Sci. USA, 1995. 92: 10277.

Qu A and Leahy DJ: The role of the divalent cation in the structure of the I domain from the CD11a/CD18 integrin. Structure, 1996, 4: 931.

Radford KJ, Thorne RF and Hersey P: CD63 associates with transmembrane 4 superfamily members, CD9 and CD81, and with beta 1 integrins in human melanoma. Biochem. Biophys. Res. Commun. 1996, 222: 17784.

Randi AM and Hogg N: I-domain of β2 integrin lymphocyte function associated antigen-1 contains a binding site for ligand intercellular adhesion molecule-1. J. Biol. Chem. 1994, 269: 12395.

Redkar V and Kenkare U: Effect of ligands on the reactivity of essential sulfhydryls in brain hexokinase. Possible interaction between substrate binding sites. Biochemistry, 1975, 14: 4704.

Reszka AA, Hayashi Y and Horwitz AF: Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. 1992, 117: 1321.

Reuvers F, Boer P and Styen-Parve EP: The effect of bacitracin on the formation of polyprenol derivatives in yeast membrane vesicles, Biochem. Biophys. Res. Commun. 1978, 82: 800.

Ridley A and Hall A: Distinct patterns of actin organization regulated by the small GTP-binding proteins Rac and Rho. Cold. Spring Harb. Symp. Quant. Biol. 1992, 57: 661.

Richardson A and Parsons JT: A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. Nature, 1996, 380: 538.

Rohwedel J, Guan K, Zuschratter W, Jin S, Ahnert-Hilger G, Furst D, Fassler R and Wobus AM: Loss of β1 integrin function results in a retardation of myogenic, but an asscleration of neuronal differentiation of embryonic stem cells in vitro. Devel. Biol. 1998, 201: 167.

Roth RA: Bacitracin: an inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase. Biochem. Biophys. Res. Commun. 1981, 98: 431.

Rothlein R and Springer TA: The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. 1986, 163: 1132.

Rothlein R, Czajkowski M, O'Neill MM, Marlin SD, Mainolfi e and Merluzzi VJ: Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. J Immunol. 1988, 141: 1665.

Rubinstein E, Le Naour F, Billard M, Prenant M and Boucheix C: CD9 antigen is an accessory subunit of the VLA integrin complexes. Eur. J. Immunol. 1994, 24: 3005.

Rubinstein E, Le Naour F, Lagaudriere-Gesbert C, Billard M, Conjeaud H and Boucheix C: CD9, CD63, Cd81 and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. Eur. J. Immunol. 1996, 26: 2657.

Ruiz P, Wiles MV and Imhof BA: Alpha6 integrins participate in pro-T cell homing to the thymus. Eur. J. Immunol. 1995, 25: 2034.

Ruoslahti E and Pierschbacher MD: Arg-Gly-Asp: a versatile cell recognition signal. Cell 1986, 44: 517.

Ryser HJP, Mandel R and Ghani F: Cell surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in Chinese hamster ovary cells. J. Biol. Chem. 1991, 266: 18439.

Ryser HJP, Levy EM, Mandel R and diSciullo GJ: Inhibition of human immunodeficiency virus infectin by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. 1994, Proc. Natl. Acad. Sci. USA. 1994, 91: 4559.

Sadhu C, Masinovsky B, Staunton DE: Differential regulation of chemoattractant-stimulated beta 2, beta 3 and beta 7 integrin activity. J. Immunol. 1998, 160: 5622.

Salmi M and Jalkanen S: How do lymphocytes know where to go: current concepts and enigmas of lymphocyte homing. Adv. Immunol. 1997, 64: 139.

Sastry SK and Horwitz AF: Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra-and intracellular initiated transmembrane signaling. Curr. Opin. Cell Biol. 1993, 5: 819.

Schaller M and Parsons J: Focal adhesion kinase and associated protein. Curr.Opin.Cell Biol. 1994, 6:705.

Schaller MD and Parsons JT: pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol. Cell Biol. 1995, 15 (5): 2635.

Scherle P, Behrens T and Staudt LM: Ly-GDI, a GDP-dissociation inhibitor of the RhoA GTP-binding protein, is expressed preferentially in lymphocytes. Proc. Natl. Acad. Sci. USA. 1993, 90: 7568.

Schlaepfer D, Hanks S, Hunter T and van-der-Geer P: Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. Nature 1994, 371: 768.

Schwartz MA, Brown EJ and Fazeli B: A 50 Kda integrin-associated protein is required for integrin regulated calcium entry in endothelial cells. J. Biol. Chem. 1993, 268(27): 19931.

Schwartz MA, Schaller MD and Ginsberg MH: Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell dev. Biol. 1995, 11: 549.

Sharma CP, Ezzell RM and Arnaout MA: direct interactin of filamin (ABP-280) with the beta 2-integrin subunit CD18. J. Immunol. 1995, 154 (&): 3461.

Shaw LM and Mercurio AM: Regulation of $\alpha 6\beta 1$ integrin laminin receptor function by the cytoplasmic domain of the $\alpha 6$ subunit. J. Cell Biol. 1993, 123: 1017.

Shaw AR, Domanska A, Mak A, Gilchrist A, dobler K, Visser L, Poppema S, fliegel L, Letarte M and Willett BJ: ectopic expression of human and feline CD9 in a human B cell line confers beta 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. J. Biol. Chem. 1995, 270(41): 24092.

Shimizu Y, Van Seventer GA, Horgan KJ and Shaw S: Regulated expression and binding of three VLA (beta 1) integrin receptors on T cells. Nature 1990, 345: 250.

Shimizu Y and Mobley JL: Distinct divalent cation requirements for integrin-mediated CD4+ T lymphocyte adhesion to ICAM-1, fibronectin, VCAM-1 and invasin. J. Immunol. 1993, 151: 4106.

Simon KO, Nutt EM, Abraham DG, Rodan GA and Duong LT: The α2β1 integrin regulates α5β1-mediated cell migration toward fibronectin. J. Biol. Chem. 1997, 272: 29380.

Smith JW and Cheresh DA: Integrin (alpha v beta 3)-ligand interaction. Indentification of a heterodimeric RGD binding site on the vitronectin receptor. J. Biol. Chem. 1990, 265 (4): 2168.

Sonnenberg A: Integrins and their ligands. Current Topics in Microbiolgy and Immunology, 1993, 184: 7.

Spencer JP, Kang MS and Elbein AD: Inhibition of lipid-linked saccharide synthesis by bacitracin. Arch. Biochem. Biophys. 1978, 190: 829.

Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994, 76: 301.

Spinardi L, Ren Y, Sanders R and Giancotti FG: The β4 cytoplasmic domain mediates the interaction of α6β4 integrin with the cytoskeleton of hemidesmosomes. Mol. Biol. Cell 1993, 4: 871.

Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Behrendtsen O, Pedersen RA and Damsky CH: Deletion of β1 integrins in mice results in inner cell mass failure and preimplanation lethality. Genes Dev. 1995, 9: 1883.

Stewart M, Thiel M and Hogg N: Leukocyte integrins. Curr. Opin. Cell. Biol. 1995, 7: 690.

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

Stewart MP, McDowall A and Hogg N: LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca++-dpendent protease, Calpain. J. Cell Biol. 1998, 140: 699.

Strauch UG, Lifka A, GoBlar U, Kilshaw PJ, Clements J and Holzmann B: Distinct binding specificities of integrins alpha4 beta7 , alpha4 beta 1 and alphaIEL beta7. Int. Immunol. 1994, 6: 263.

Stupack DG, Stewart S, Carter WG, Wayner EA and Wilkins JA: B lymphocyte fibronectin receptors: expression and utilization. Scand. J. Immunol. 1991, 34: 761.

Stupack DG, Shen C and Wilkins JA: Induction of αvβ1 integrin-mediated attachment to extracellular matrix in β1 integrin (CD29)-negative B cell lines. Exp. Cell Res. 1992, 203: 443.

Stupack DG, Shen CX and Wilkins JA: Control of lymphocyte integrin function: Evidence for multiple contributing factors. Cell. Immunol. 1994, 155: 237.

Suzuki S and Naitoh Y: Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. EMBO. J. 1990, 9 (3): 757.

Tager M, Kroning H, Thiel U, Ansorge S: Membrane-bound protein disulfide isomerase (PDI) is involved in regulation of surface expression of thiols and drug sensitivity of B-CLL cells. Exp. Hematol. 1997, 25: 601.

Takada Y and Puzon W: Identification of a regulatory region of the integrin β1 subunit using activating and inhibiting antibodies. J. Biol. Chem. 1993. 268: 17597.

Takada Y, Kamata T, Irie a, Puzon-Mclaughlin W and Zhang XP: Structural basis of integrin-mediated signal transduction. Matrix biology. 1997, 16: 143.

Tamura RN, Cooper HM, Collo G and Quaranta V: Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. Proc. Natl. Acad. Sci. USA. 1991, 88 (22): 10183.

Taooka Y, Chen J, Yednock T and Sheppard D: The integrin α9β1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. J. Cell Biol. 1999, 145: 413.

Tidswell M, Pachynski R, Wu S, Qiu S, Dunham E, Cochran N, Briskin M, Kilshaw P, Lazarovits A, Andrew D, Butcher E, Yednock T and Erle D: Structure-function analysis of the integrin beta-7 subunit. J. Immunol. 1997, 159: 1497.

Tozer EC, Hughes PE and Loftus JC: Ligand binding and affinity modulation of integrin. Biochem. Cell Biol. 1996, 74: 785.

Tsuji K and Robertson JH: Improved high performance liquid chromatographic method for polypeptide antibiotics and its application to study the effects of treatments to reduce microbial levels in bacitracin powder. J. Chromatogr. 1975, 112: 663.

Tuckwell D and Humphries M: A structure prediction for the ligand-binding region of the integrin β subunit: evidence for the presence of a von Willebrand factor A domain. FEBS Lett. 1997, 400: 297.

Ugarova TP, Zamarron C, Veklich Y, Bowditch R, Ginsberg MH, Weisel JW and Plow EF: Conformational transitions in the cell binding domain of fibronectin. Biochemistry. 1995, 34: 4457.

Umehara H, Minami Y, Domae N and Bloom ET: Increased processing of lymphocyte function-associated antigen-1 in human natural killer cells stimulated with IL-2. Int-Immunol. 1994, 6(7): 1071.

Valmu L, Autero M, Silijander, Patarroyo P and Gahmberg CG: Phosphorylation of the beta-subunit of CD11/CD18 integrins by protein kinase C correlates with leukocyte adhesion. Eur. J. Immunol. 1991, 21: 2857.

Valmu L, Hilden TYJ, van Willigen G and Gahmberg CG: Characterization of β2 (CD18) integrin phosphorylation in phorbol ester-activated T lymphocytes. Biochem J. 1999, 339: 119.

Vennegoor C, van de Wiel-van Kemenade E Huijbens RJ, Sanchez-Madrid F, Melief C and Figdor CG: Role of LFA-1 and VLA-4 in the adhesion of cloned normal and LFA-1 (CD11/CD18)-deficient T cells to cultured endothelial cells. Indication for a new adhesion pathway. J. Immunol. 1992, 148: 65.

Von Andrian U, Berger E, Ramezani L, Chambers J and Arfors K: In vivo behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. J. Clin. Invest. 1993, 91: 2893.

Wang D, Zhou GH, Birkenmeier TM, Gong J, Sun L, Brattain MG: Autocrine transforming growth factor beta 1 modulates the expression of integrin alpha 5 beta 1 in human colon carcinoma FET cells. J. Biol. Chem. 1995, 270: 14154.

Wang R, Peterson J, Aster RH and Newman PJ: Disruption of a long range disulfide bond between residurs Cys406 and Cys655 in glycoprotein IIIa does not affect the function of platelet glycoprotein IIb-IIIa. Blood, 1997, 90(4): 1718.

Wantanabe G, Nakanishi I, Yamashita K, Hayakawa T and Okada Y: Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation and cellular invasion. J. Cell Sci. 1993, 104: 991.

Wary KK, Mainiero F, Isakoff SJ, Marcantonio EE and Giancotti FG: The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell. 1996, 87 (4): 733.

Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA and Carter WG: Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. 1989, 109: 1321.

Weber C, Katayama J and Springer TA: Differential regulation of beta 1 and beta 2 integrin avidity by chemoattractants in eosinophils. Proc. Natl. Acad. Sci. U. S. A. 1996, 93: 10939.

Weissman IL and Cooper MD: How the immune system develops. Sci. Am. 1993, 269(3): 33.

Wennenberg K, Lohikangas L, Gullberg D, Pfaff M, Johansson S and Fassler R: Beta1 integrin dependent and –independent polymerization of fibronectin. J. Cell. Biol. 1996, 132(1-2): 227.

Wilcox DA, Wautier JL, Pidard d and Newman PJ: A single amino acid substitution flanking the fourth calcium binding domain of alpha Iib prevents maturation of alpha Iib beta 3 integrin complex. J. Biol. Chem. 1994, 269 (6): 4450.

Williams EC, Janmey PA, Ferry JD and Mosher DF: Conformational states of fibronectin. Effects of pH, ionic strength and collagen binding. J. Biol. Chem. 1982, 257: 14973.

Willian HJ et al: The inner world of cell adhesion: integrin cytoplasmic domains. Trends Cell Biol. 1994, 4: 109.

Wilkins JA, Li AL, Ni HY, Stupack DG and Shen CX: Control of beta 1 integrin function. Localization of stimulatory epitopes. J. Biol. Chem. 1995, 271: 3046.

Wilkins JA, Stupack DG, Stewart S and Caixia S: Beta 1 integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. Eur. J. Immunol. 1991, 21: 517.

Winn RK and Harlan JM: CD18-independent neutrophil and mononuclear leukocyte emigration into the peritoneum of rabbits. J. Clin. Invest. 1993, 92: 1168.

Xiao J, Messinger Y, Jin J, Myers DE, Bolen JB and Uckun FM: Signal transduction through the beta1 integrin family surface adhesion molecules VLA-4 and VLA-5 of human B cell precursors activates CD19 receptor-associated protein-tyrosine kinases. J. Biol. Chem. 1996, 271: 7659.

Xu H, Gonzalo JA, Pierre Y, Williams IR, Kupper TS, Cotran RS, Springer TA and Gutierrez-Ramos JC: Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J. Exp. Med. 1994, 180: 95.

Yan B, Hu DD, Knowles SK and Smith JW: Probing chemical and conformational differences in the resting and active conformers of platelet integrin alphaIIbbeta3. J. Biol. Chem. 2000, 275: 7249.

Yan B and Smith JW: A redox site involved in integrin activation. J. Biol. Chem. 2000, Sep. 18: in press.

Yang JT, rayburn H and Hynes RO: Embryonic mesodermal defects in alpha 5 integrin-deficient mice. Development. 1993, 119 (4): 1093.

Yednock TA, Cannon C, Vandevert C, Goldbach EG, Shaw G, Ellis DK, Liaw C, Fritz LC and Tanner LI: Alpha 4 beta 1 integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. J. Biol. Chem. 1995, 270: 28740.

Ylanne J, Huuskonen J, O'Toole TE, Ginsberg MH, Virtanen I and Gahmberg CG: Mutation of the cytoplasmic domain of the integrin β3 subunit. J. Biol. Chem. 1995, 270: 9550.

Yoshimori T, Semba t, Takemoto H, Akagi S, Yamamoto a and Tashiro Y: Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. J. Biol. Chem. 1990, 265: 15984.

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, Giannetti A, De Luca M: Transforming growth factor-beta1 modulates beta1 and beta5 integrin receptors and induces the do novo expression of alphaV beta6 heterodimer in normal human keratinocytes: implications for wound healing. J. Cell Biol. 1995, 129: 853.

Zheng M, Fang H, Hakomori S: Functional role of N-glycosylation in alpha5 beta1 integrin receptor . De N-glycosylation induces dissociation or altered association of alpha5 and beta1 subunits and concomitant loss of fibronectin binding activity. J Biol. Chem. 1994, 269: 12325.

Zhidkova NI, Belkin AM and Mayne R: Novel isoform of beta 1 integrin expressed in skeletal and cardiac muscle. Biochem. Biophys. Res. Commun. 1995, 214 (1): 279.

Zhou L, Lee DHS Plescia J, Lau CY and altieri DC: Differential ligand binding specificities of recombinant CD11b/CD18 integrin I-domain. J. Biol. Chem. 1994, 269: 17075.