THE UNIVERSITY OF MANITOBA

THE FUNCTIONAL AND STRUCTURAL ANALYSIS OF INTEGRIN β_1
BY MAPPING THE EPITOPES OF STIMULATORY mAbs OF INTEGRIN β_1

BY
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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LIST OF ABBREVIATIONS

aa	amino acid
Ag	antigen
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
ddH$_2$O	double distilled water
cDNA	complementary deoxyribonucleic acid
Da	dalton
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DPM	dimethylpimelimidate
E.coli	Escherichia coli
EDTA	ethylene diamine tetra-acetic acid
Fig.	figure
g	gram
Ig	immunoglobulin
kDa	kilodalton
Kbp	kilobase pair
MAb	monoclonal antibody
min.	minute

NBT	nitroblue tetrazolium chloride
NCM	nitrocellulose membrane
NP-40	nonidet-p40
PEG	polyethylene glycol
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
TFMS	trifluoromethanesulfonic acid
Tris-Cl	tris hydrochloride
Tween-20	polyoxyethylaminomethane
ug	microgram
ml	milliliter
ul	microliter
M	molar
mM	millimolar

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Abstract

Integrins are cell surface receptors that mediate cell-cell and cell-matrix adhesion. Members of the β_1 integrin subfamily recognize multiple ligands such as fibronectin, laminin, collagen and play important roles in many biological events. The binding capacity of integrins to their ligands is regulated by cells. The β_1 subunit plays an important role in regulating β_1 integrin binding capacity. In previous studies, a group of monoclonal antibodies were produced against the human β_1 subunit and identified as stimulatory antibodies that can up-regulate integrin ligand binding capacity. In this study, epitope mapping of these antibodies was used to identify regions of the β_1 subunit involved in this function. To achieve this, a β_1 epitope library which was expressed as a fusion protein in an E.coli host strain was generated and screened with a panel of stimulatory antibodies. The epitopes were identified by sequencing the inserts of positive clones to which the antibody reacted. I found that there are two distinct molecular regions influencing up-regulation of β_1 integrin mediated attachment. The epitopes of the stimulatory mAbs JB1B, B3B11 and 21C8 are located on the membrane proximal region (648-705aa) of β_1 integrin. The epitope of the other stimulatory monoclonal antibody N29 is located on the amino terminal region (1-60aa) of β_1 integrin. In the study of functional regulation of β_3 integrin, the homologous regions (residue 1-6 and residue 602-690) have been shown to be the sites recognized by stimulatory antibodies to ligand-induced binding sites. This study indicates that there are multiple regulatory regions on the β_1 subunit and suggest structural and functional homology between the locations of β_1 and β_3 regulatory sites.

Some functions of integrin are known to be affected by post-translational modification. Through the study of deglycosylation of the β_1 native protein, we found that B44, a stimulatory antibody of β_1 integrin recognized a carbohydrate epitope or partial carbohydrate epitope. This suggests that carbohydrates may also affect the binding capacity of β_1 integrin.

INTRODUCTION

I. .CELL ADHESION MOLECULES

Cellular adhesion and recognition mechanisms are among the most basic requirements for the evolution of multicellular organisms. During the development of an embryo, cellular adhesion proteins can impart position-specific information which guides cell migration, localization, and the transfer of information between cells. As cells are triggered to differentiate to form tissue or organs, adhesion proteins help to maintain the organization and integrity of the body.

There are two principal types of cell adhesion; cell-cell adhesion and cell-extracelluar matrix adhesion. Morphological, cell biological and biochemical studies all indicate that even a single cell type utilizes multiple molecular mechanisms in adhering to other cells and to extracelluar matrix. The cell-cell and cell-extracelluar interactions are mediated by several families of adhesion molecules which have been characterized molecularly, including cadherins, selectins, members of the immunoglobulin superfamily and integrins.

1.CADHERINS

Many cells of multicellular organisms are able to distinguish different cell types, generally adhering preferentially to cells of their own type. Early developmental studies have shown that embryonic cells can reaggregate after dissociation by enzymatic treatment and reform tissue-like structures. Furthermore, when cells from different tissues were mixed, they sorted out (Holtfreter, 1948;

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steinberg, 1970). This selective cell adhesion or cell sorting is fundamental to controlling the development and maintenance of tissues and is observed in a wide variety of developmental and pathological events (for example, egg-sperm interactions, neuronal connections, and lymphocyte homing). The cadherin family of cell-cell adhesion receptors is central to these processes (Takeichi, 1991). Cadherins are a family of cell surface glycoproteins which are expressed widely in the solid tissues of both vertebrates and invertebrates (Oda,1990). They are Ca^{2+}-dependent cell-cell adhesion molecules which hold cells together by a homophilic interaction (molecules on one cell bind to other molecules of the same kind on adjacent cells) (Takeichi, 1991). To date, more than a dozen cadherins have been identified (Redies,1995). They can be grouped into at least four classes based on their molecular structure: 1) the classical cadherins which are N-, R-, E-, P-,M-, and T-,cadherin (Pouliot, 1992; Suzuki, 1991; Tanihara, 1994), 2) the numbered cadherins, which are cadherin 5 to cadherin-12 (Suzuki, 1991;Tanihara, 1994), 3) the protocadherins (Sano, 1993), 4) the desmosomal cadherins (Dalseg, 1993; Grunwald, 1993). The cadherins typically consist of five tandemly repeated extracelluar domains, a single membrane-spanning segment and a cytoplasmic domain (Takeichi,1990; Blaschuk, 1994; Overduin, 1995). The N-terminal extracelluar domains mediate cell-cell contact (Nose,1992) while the cytoplasmic region interacts with the cytoskeleton through the catenins (Kemler,1993). Cadherins play an important role in regulating morphogenesis through selective adhesion (Takeichi, 1991).

2.SELECTINS

Selectins are lectin-like cell surface glycoproteins that have been implicated in playing a crucial role in the many steps in lymphocyte recirculation and inflammatory responses(Mackay, 1992;

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Picker,1992). So far, there are three known selectins, termed L-, E-, and P-selectins. They have a common structural organization composed of five distinguishable domains: 1) a lectin domain at the extreme N-terminus which contains some of the Ca^{2+} binding sites (Weis, 1991); 2) an EGF domain; 3) a domain of variable length containing a series of repeating regions about 60 amino acids in size that are related to complement-regulatory protein; 4) a transmembrane domain; 5) a short cytoplasmic domain (Cummings, 1992). Selectins are expressed by leukocytes, platelets and endothelia and function in cell adhesion events. L-selectin is required for homing of naive T-cells to PLN (peripheral lymph nodes) (Hamann, 1991). and is also involved in the binding and extravasation of neutrophils in inflammatory sites (Watson, 1991). The activated endothelium presents a ligand for L-selectin that mediates the adhesion of lymphocytes and leukocytes to the endothelium and leukocyte rolling (Springer, 1991). P-selectin is stored in the α granules of platelets and the Weibel-Palade bodies of endothelial cells (McEver, 1991; Hsu-Lin, 1984) and is rapidly translocates to the plasma membrane upon activation (such as exposed to thrombin or oxygen radicals) (Patel, 1991). P-selectin is an important mediator of leukocyte rolling. The other member of the selectin family is E-selectin. It is not stored, but requires de novo synthesis following stimulation and is expressed on the membrane of endothelial cells only under inflammatory conditions. The function of E-selectin in leukocyte rolling in vivo is controversial (Olofsson, 1994; Ley, 1995).

3.IMMUNOGLOBULIN SUPERFAMILY

The immunoglobulin superfamily is characterized by the presence of a common structural feature, the immunoglobulin homology domain. They are found in species from insects to human and play an essential role in inflammation and immunoresponses (Springer, 1990; Dustin, 1991).Some members

in this family include; ICAM-1 (intercellular cell adhesion molecule-1), ICAM-2, ICAM-3, VCAM-1 (vascular cell adhesion molecule-1), and PECAM-1 (platelet endothelial cell adhesion molecule-1). The first four serve as endothelial ligands for leukocytes.

The three known ICAMs (ICAM-1,-2, and -3) have five, two, and five Ig domains respectively, and ICAM-1 and ICAM-3 are the most closely related.(de Fougerolles, 1991; Vazeux, 1992). The ICAMs all bind to the leukocyte integrin $\alpha_L\beta_2$ and ICAM-1 also binds to an additional integrin $\alpha_M\beta_2$ (Diamond,1991; Staunton, 1990). The different expression profile of ICAMs support that they have different functions. ICAM-1 is expressed at low levels under normal conditions and is strongly up-regulated by cytokines IL-1, TNF, or interferon-γ after several hours stimulation on many cell types, including leukocytes, endothelial cells, keratinocytes and fibroblasts (Rohlein,1988; Doussis-Anagnostopoulou, 1993). This may increase cell-cell interaction and leukocyte extravasation at inflammatory sites. ICAM-2 is constitutively expressed by all leukocytes and endothelial cells at low levels and may be important for leukocyte trafficking in uninflamed tissues, as in lymphocyte recirculation. ICAM-3 is constitutively highly expressed by leukocytes and absent on endothelium. ICAM-3 is the dominant ligand for $\alpha_L\beta_2$(Campanero, 1993) and provides a costimulatory signal for cell proliferation (de Forgerolles, 1994). All the ICAMs play important roles in directing the migration and colocalization of leukocytes in inflammatory sites. In addition, ICAM-3 appears to be the major ligand for $\alpha_L\beta_2$ in the primary phase of an immune response and functions as signal transducer via β_1 integrin and β_2 integrin pathway (Hernandez-Caselles,1993).

VCAM-1 is an adhesion molecule expressed in vitro on cytokine-activated endothelium (Springer,

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1990) and in vivo on inflamed vascular endothelium (Rice,1990). The major form of VCAM-1 in humans contains seven Ig domains and binds with integrin $\alpha_4\beta_1$ through the first and fourth Ig domains (Osborn, 1992; Pepinsky, 1992). VCAM-1 is absent on resting endothelial cells, but is strongly induced by IL-1 and TNF (Masinovsky,1990). VCAM-1 is also a regulator of lymphocyte extravasation at sites of inflammation.

CD31 (also known as platelet endothelial cell adhesion molecule-1 [PECAM-1]) is a member of the immunoglobulin family and has six Ig domains (Newman, 1990; Simmons, 1990). CD31 is a constitutively and abundantly expressed surface glycoprotein on vascular endothelium (Newman, 1992), platelets, monocytes, and neutrophils (Stockinger, 1992; Bird, 1993) and plays a role in the transendothelial migration of monocytes and neutrophils (MUller, 1993).

4. INTEGRINS

Integrins are the major family of cell surface receptors which mediate attachment to the extracelluar matrix and cell-cell interactions. Integrin-mediated cell adhesion plays a critical role in the regulation of many cellular functions, such as embryonic development, tumour cell growth and metastasis, programmed cell death(apoptosis), haemostasis, leucocyte homing, and activation, clot retraction and the response of cells to mechanical stress (DeSimone, 1994; Juliano, 1993; Schwartz, 1994; Shattil, 1994). The integrins that function as extracelluar matrix receptors can also direct the assembly of extracelluar matrix by cells. The name of integrin was coined for these receptors because they are thought to allow for the interaction between the internal cytoskeleton of a cell and the extracelluar matrix. The details about the integrin's classification, structure, function, and functional regulation

will be given later in this thesis.

II. THE COOPERATION AND INTERACTION OF DIFFERENT ADHESION MOLECULES IN BIOLOGICAL EVENTS.

The organization of animal cells in the differentiated organs and tissues has been postulated to depend on cell surface interactions both with molecules on the surface of other cells and with the ECM. Localization of cells can thus mainly be driven by the interplay between interactions with cell-surface and matrix molecules that regulate adherence, and chemoattractant gradients that direct cell migration. The different adhesion molecules cooperate and interact with each other to achieve a physiologic function. The mechanisms for regulating adhesion are particularly richly illustrated by the adhesion receptors of the immune system.

To patrol the body effectively for infectious organisms, the cells of immune system must both circulate as non-adherent cells in the blood and lymph and migrate as adherent cells through tissue and rapidly accumulate at sites of injury and infection, and then subsequently return into the circulation. Abnormal accumulation of immune cells in unwanted sites might lead to the development of autoimmune disease and cell-mediated tissue destruction. There are three families of adhesion receptors that mediate lymphocyte homing to lymphoid organs such as peripheral lymph nodes (PLN) and Peyer's patches and localization of leukocytes in inflammatory sites. They are 1) the immunoglobulin superfamily, which includes the antigen specific receptors of T and B lymphocyte,

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2) the integrin family, which is important in dynamic regulation of adhesion and migration, and 3) the selectin family, which are prominent in lymphocyte and neutrophil interactions with vascular endothelium. These three families of adhesive receptors cooperate and interact in cell adhesion events (Butcher, 1991; Springer, 1994).

Normally, leukocytes adhere poorly to resting endothelial cells. But when inflammation occurs , the local endothelium is dramatically modified by cytokines, such as IL-1and TNF which are mainly produced by macrophages stimulated by microbial products and IFN-γ, which is released by natural killer cells and T cells that have met their specific antigen (Imhof,1995). These cytokines induce the exocytosis (P-selectin) and/or biosynthesis (E-selectin) of selectins on the surface of activated endothelial cells. The selectins bind carbohydrates (their ligands) on the leukocyte surface and cause the leukocytes to tether to the vessel wall and roll along the vessel wall through labile adhesion (Lanrence, 1991; Ley, 1991; von Andrian, 1991; Mayadas, 1993). Tethering brings leukocytes into proximity with chemoattractants that are displayed on or released from the endothelial lining of the vessel wall. The chemoattractant receptors on the leukocytes, which are G protein-coupled receptors,bind with chemoattractants that are displayed on or released from the endothelial cell and transduce signals that activate integrin adhesiveness (Springer, 1994). Five integrins play critical roles in the interaction of leukocytes with endothelial cells. They are $\alpha_L\beta_2$ $\alpha_M\beta_2$ $\alpha_X\beta_2$ $\alpha_4\beta_1$ $\alpha_4\beta_7$ (Springer, 1990; Aemler, 1990; Berlin, 1993). The activated integrins which are inactive on the resting circulating leukocyte surface bind with their counter-receptor Ig superfamily member such as ICAMs, VCAM-1 and achieve strong adhesion and arrest (Springer,1990; Landis, 1993; Diamond, 1993). The importance of the leukocyte integrins is illustrated in the leukocyte adhesion deficiency (LAD) in

which they are deficient because of mutation in the common β_2 subunit (Kishimoto, 1989; Anderson, 1987). Patients have recurring infections, often fatal in childhood unless they are treated by bone marrow transplantation. The binding of the β_1 subfamily integrins to extracelluar matrix protein contributes to invasion and migration into underlying tissues and controls leukocyte localization in inflammatory sites. So this complex adhesion event requires a cascade of adhesion, involving several different adhesion receptor families.

III. CLASSIFICATION OF INTEGRINS

Integrins are a family of structurally related glycoproteins. They are all α β heterodimers. So far fifteen different α subunits and eight different β subunit have been identified. These subunits selectively heterodimerize to form one of twenty-one complexes and these complexes functionally behave as receptors for different ECM proteins (Clark, 1995).

The integrin family can be subdivided into eight subfamilies based on the β subunit which is shown in Table 1(Ruoslahti, 1994). The β_1 integrin subfamily is the largest and most widely distributed. The β_1 subunit can form α β heterodimers with nine different α subunits (α_{1-8} and α_v) and form the receptors for fibronectin, lamimin, various collagens and vitronectin (Sonnenberg 1993, Hynes 1992, Kieffer 1990). The second subfamily is composed of one of the four α subunits (α_L, α_M, α_X, α_D) complexed with the β_2 subunit. This subfamily forms leucocyte-specific receptors and plays an important role in guiding extravasation and migration of leucocytes at inflammatory sites (Larson, 1990).The β_2 integrins mainly mediate cell-cell, rather than cell-matrix interactions by binding to

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specific ligands on other cells. The cytoadhesions ($\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$) form the third subfamily of integrins. They are found on a variety of cells, including blood platelets, and they bind several matrix protein, including fibrinogen,and play an important function in haemostasis and thrombosis (Phillips, 1988). The β_7 subfamily is composed of $\alpha_4\beta_7$ and $\alpha_E\beta_7$. They are expressed on a subset of lymphocytes which colonize the gut and gut-associated lymphoid tissues(Holzmann, 1989. Hu, 1992). Neuhaus, 1991. Schweighoffer,1993) and mediate lymphocyte homing to Peyer's patches(Imhof and Dunon, 1995). The functions of the rest of the integrin subfamilies are still being characterized.

Fig.1

THE CLASSIFICATION OF INTEGRIN
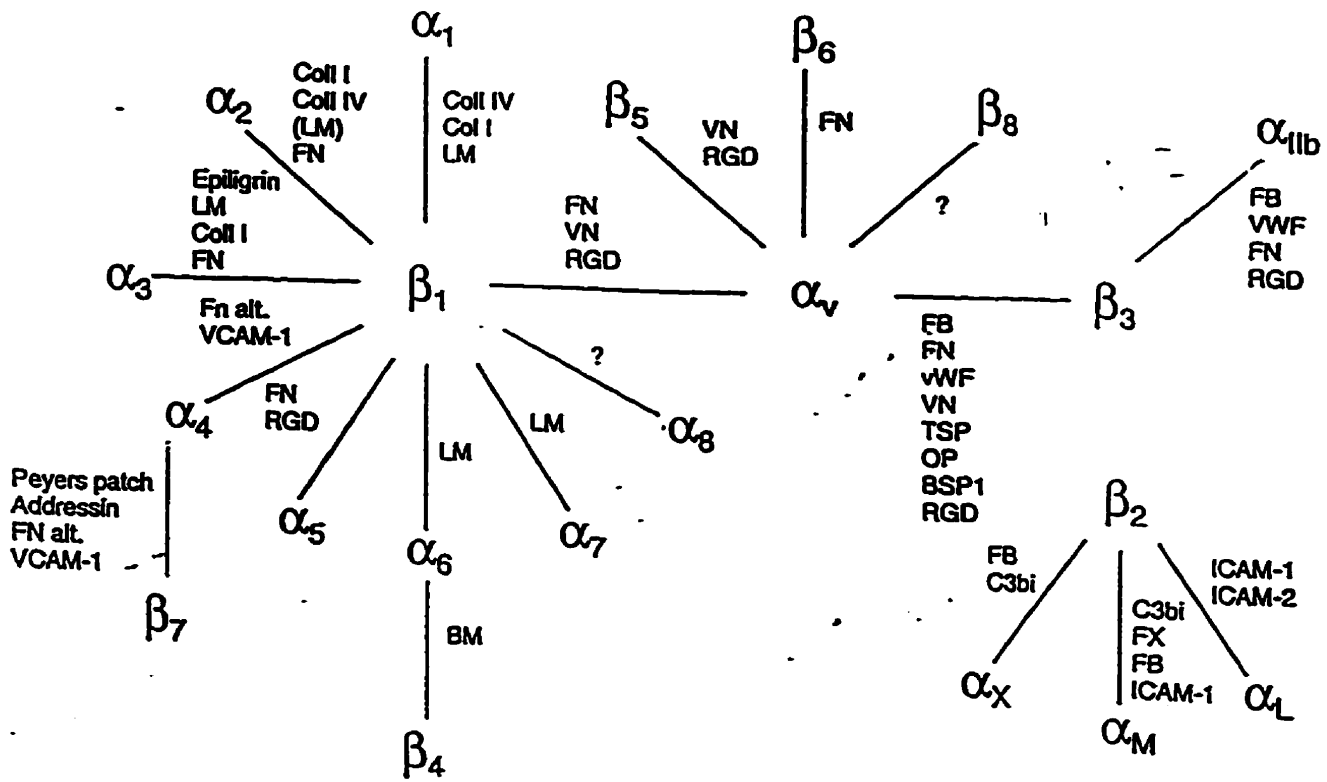
This table is copied from reference (Ruoslahti,1994)

VI. STRUCTURE OF INTEGRIN

Integrins are crucially important receptor proteins because they are the main way that cells both bind and respond to the extracellular matrix. They are a family of transmembrane receptor molecules. All integrins are α β heterodimers and both subunits can be divided into three domains. From N-terminal they consist of a large extracelluar domain, a short single hydrophobic transmembrane domain and a short cytoplasmic domain (Fig.2) (β_4 is an exception which has over a 1000 amino acids in it's cytoplasmic domain) (Hynes,1992). The extracelluar domains of the two subunits non-covalently associate to form α β heterodimers. The association of α and β subunits does not rely on the transmembrane and cytoplasmic domains (Solowska,1991) but is promoted by divalent cations (Ginsberg,1988). Newly synthesized receptor molecules are transported to the cell surface after the subunits are combined (Cheresh and Spiro,1987). Electron microscopic images of several integrins show a globular head apparently comprising parts of both subunits and two stalks extending to the lipid bilayer (Carrel, 1985; Nermut, 1988). The α subunits all have large extracelluar domains which contain three to four divalent cation binding domains and this region also contributes to the ligand-binding domain (Hynes, 1992). The β subunits all contain a four-fold repeat of a cystine-rich domain which is formed by internal disulfide bonds (Calvete, 1991). The N-terminal 40-50 Kd is tightly folded with internal disulfide loops and contributes to the ligand-binding domain (Hynes, 1992). Chemical crosslinking studies show that the ligand binds in proximity to the N-terminal cation binding domain of the α subunit and to a 100 amino acid segment in the β subunit, the later which is critical for ligand binding as demonstrated by mutagenesis studies (Hynes,1992). Thus the N-terminal domains of α β subunits associate to form a ligand-binding head on each integrin. This ligand-binding head is linked

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by two stalks which span the membrane and fix the receptor on the cell surface. The cytoplasmic

domain of both subunits are short except for β_4

Fig. 2 Structural features of integrin receptors

(a) a shows the overall shape, as deduced from electron microscopy, as well as the putative locations of the cystine-rich repeats of the ß subunit(crosshatched) and metal-binding sites in the alpha subunit(M++). The shaded are represents the ligand-binding sites.

(b) schematizes the arrangement of the polypeptide chains with the cystine repeats internally folded and the head region of the ß subunit containing inernal disulfide loops, some but not all of which are show are shown. A disulfide bond from the middle of the ß subunit to a point close to the membrane has been proposed (Calvete, 1991) but is omitted here for clarity. Xs indicate positions of mutations(of human beta 2 or beta 3 subunits) known to affect ligand binding or alpha beta dimerization. The positions of alternatively spliced segments in Drosophila subunits are shaded. This figure is from reference (Hynes 1992).

Fig.2

THE STRUCTURE OF INTEGRIN
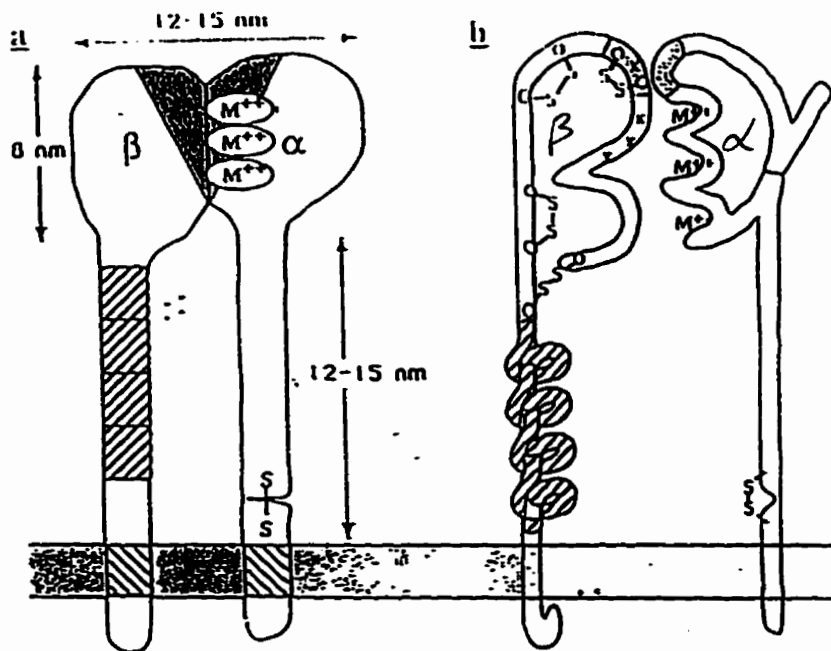
This figure is from reference (Hynes 1992)

V.FUNCTIONS OF INTEGRIN

Integrins function as transmembrane linkers which mediate the interaction between the cytoskeleton and the extracelluar matrix which is required for cells to adhere the matrix. They also function as bidirectional signalling molecules.

1. GENERAL BINDING PROPERTIES

1.1 Diversity

Most integrins are expressed widely and most cells express several integrins. Individual integrins can bind with more than one ligand and individual ligands can be recognized by more than one integrin (Hynes, 1992). For example, T cell can express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_L\beta_2$. Integrin $\alpha_2\beta_1$ can bind to collagen and laminin. Collages can react with $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$ (Sonnenberg, 1993). However, different integrins that recognize the same ligand may mediate different functions. For example, both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ bind to vitronectin, but only $\alpha_v\beta_3$ promotes migration (Leavesly, 1992). The cytoplasmic domain is clearly important in such postligand binding events. This has been demonstrated by constructing chimeric integrins which contain the cytoplasmic domains of various other integrins (Chan, 1992).

1.2 Specificity

The binding specificity of a particular integrin may vary according to the cell type further increasing

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the complexity. $\alpha_2\beta_1$ is a collagen receptor on platelets and a collagen/laminin receptor on endothelial cells(Kirchofer,1990). Therefore, integrins mediate an extraordinary variety of biological processes by using different integrins to bind to different extracelluar matrix proteins (cell-ECM adhesion) and integral membrane proteins of the immunoglobulin superfamily (cell-cell direct adhesion).

2. TRANSMITTING SIGNALS INTO OR OUT OF CELLS

The interaction of cells with the extracelluar matrix regulates cell shape, motility, growth, survival, differentiation and gene expression, through integrin-mediated signal transduction (Damsky,1992; Hynes,1992; Clark,1995). Integrins function as signal transducers and regulators behaviour in nearly every cell type. Perhaps the most-studied function is cell cycle progression. Replication of normally adherent cells such as fibroblasts and endothelium requires anchorage to a solid substratum (Stoker, 1986; Folkman 1978).

The activation of leukocytes and platelets is also regulated by adhesion to the ECM. A well documented example is $\alpha_{IIb}\beta_3$ integrin which is the major integral platelet membrane protein (17% of the platelet membrane protein mass) (Phillips, 1988). When $\alpha_{IIb}\beta_3$ integrin is activated by its ligands such as fibrinogen, there is tyrosine phosphorylation event is induced (Shattil, 1991). Recently, there is additional evidence to indicate that tyrosine phosphorylation is a common response to integrin engagement in many cell types including fibroblasts, carcinoma cells, and leucocytes (Juliano, 1993; Hynes, 1992; Arroyo, 1994).

Integrins have been identified as the signal transducing molecules that mediate control of gene expression by the ECM. Thus, adhesion of T cells to fibronectin via $\alpha_4\beta_1$ and $\alpha_5\beta_1$ or to laminin via $\alpha_6\beta_1$ provides a co-stimulatory signal for CD3 dependent T cell proliferation (Shimizu, 1990); treatment with antibodies to β_1 integrin results in the expression of inflammatory mediator genes in monocytes (Yurocko, 1992).

Apotosis or programmed cell death is potentially growth-related function modulated by integrins. Just as deprivation of growth factors can lead to apotosis of cells in culture, maintaining cells in suspension without contact with ECM proteins can also trigger programmed cell death. Integrin $\alpha_5\beta_1$ mediated binding of fibronectin to leukaemia cells inhibits growth factor dependent proliferation and induces apotheosis (Sugahara, 1994).

3. INTERACTION WITH THE CYTOSKELETON

Intracellularly, most integrins are linked to cytoskeleton. Following the binding of a typical integrin to its ligands in the matrix, the cytoplasmic tails of both α and β subunit bind to both talin and α-actinin through indirect linkage, involving talin, vinculin, α actinin and other molecules and initiate the assembly of a complex of intracellular attachment proteins that link the integrin to actin filaments in the cell cortex (focal contact)(Burridge, 1988; Hynes, 1992). Both the α and β subunits are though to be involved in binding to the cytoskeleton. Interfering with any of these three elements results in disruption of adhesive cell-matrix interactions. Further evidence for the importance of the cytoplasmic domain of β_1 subunit comes from data showing that mutants or isoforms altered in this

region fail to associate with focal contacts (Reszka, 1992; Balzac, 1993) and may even inhibit focal adhesion and cell spreading (Balzac, 1994). So interaction with the cytoskeleton is likely to be important in the functions mediated by integrin.

V. COMFORMATIONAL CHANGES OF INTEGRIN

The activities of integrins are regulated by the cells expressing them. Whereas the matrix-binding integrins of many cells in tissue are constantly in an adhesive-competent state, the integrins on blood cells often have to be activated before they can mediate cell adhesion. It has been demonstrated that adhesion is critical to the regulation of the distribution of vascular cells within specific tissues and organs. Unlike most other cells, most vascular cells express a wide repertoire of integrins. For example, platelets contain at least five members of the integrin family, lymphocytes seven, endothelial cells six (Andrews,1990). Vascular cells may be selectively retained within specific organs and tissue or recirculated through the blood/lymphatic system and present extremely complex adhesive properties. To meet these diverse adhesive requirements, the ligand binding properties of integrins undergo cell specific regulation. For example, in tissues stimulated by inflammatory mediators, cytokines, or in the presence of a variety of cognates of the adhesive receptor, non-adhesive circulating lymphocytes may attach to the endothelium, extravasate and migrate to the inflammatory sites. The exact mechanisms of regulation of integrin function are complex and regulation of integrins by affinity modulation is only one of the mechanisms (Smyth, 1993).

Affinity modulation of integrin is a term used to describe the phenomena that is the result of agonist-

induced conversion of integrins from a form that can not interact with ligands to one that can. It has become increasingly apparent that certain cells acquire specific adhesive properties by altering the structure of existing integrins through an unidentified series of intracellular steps that are initiated by cellular activation and occur within seconds (Smyth, 1993). Affinity modulation is a property of $\alpha_{IIb}\beta_3$ (Bennett, 1979), β_2 (Crowe, 1994), β_7, and β_1 integrins (Faull, 1993). Recently there is increasing evidence to indicate that some integrins are expressed on the cell surface in a latent form. The latent form can not interact with ligands but can convert to an activated form by altering the structure through an unidentified series of intracellular steps in the response to the stimulation of agonist, some monoclonal antibodies etc (Shimizu, 1990; Wilkins, 1991; Smith, 1993; Chan, 1991). The well characterized example is integrin $\alpha_{IIb}\beta_3$ (Kieffer and Phillips, 1990; Phillips, 1991). Integrin $\alpha_{IIb}\beta_3$ on the resting circulating platelet does not binding any of its soluble ligands and does not aggregate. Only after platelet activation by thrombin, collagen, ADP (adenosine diphosphate, thromboxane A_2 agonists, does $\alpha_{IIb}\beta_3$ become an effective receptor for soluble fibrinogen and cause thrombus formation (Bennett, 1979; Plow, 1980; Bennett, 1981; Marguerie, 1981; Phillips, 1991). This activation of platelet $\alpha_{IIb}\beta_3$ in response to thrombin is mediated by heterotrimeric G proteins (Shatti, 1987) appearing to act through protein kinase C (PKC). Integrin cytoplasmic domains are plausible substrates for PKC. β_3 but not α_{IIb} is phosphorylated in response to activators of PKC (Schwartz, Schaller and Ginsberg, 1995). This activation is also accompanied by a conformational change in $\alpha_{IIb}\beta_3$ which can be detected immunologically (Shattile, 1985; Gulino, 1990; Kouns, 1990; O'Toole, 1991; Andrieux, 1991). The mechanisms responsible for the transition to an activated state are unknown. A model of affinity modulation of integrins has been proposed (Schwartz, Schaller and Ginsberg, 1995). Cell-type-specific energy-dependent cytoplasmic signals target the integrin

cytoplasmic domains. The unidentified factor(s) responsible for these signals interact with the cytoplasmic domains to induce changes in the spatial relationships or conformations of the α and β subunit cytoplasmic tails which is presumably transmitted in some manner to the extracelluar domains.

Another piece evidence was obtained using antibody directed against ligand-induced binding sites (anti-LIBS). LIBS are the sites expressed on the cell surface receptor only after ligand binding by receptor and this causes the receptor to change conformation and expose the epitopes for anti-LIBS antibodies. In the case of $\alpha_{IIb}\beta_3$, a number of stimulatory anti-LIBS antibodies (D3GP3 (Kouns,1991), AP5 (Honda et al,1995) were generated and these antibodies only preferentially react with ligand-occupied conformers of $\alpha_{IIb}\beta_3$ which suggest there is a ligand-inducible conformational change (Frelinger, 1990). It is apparent that $\alpha_{IIb}\beta_3$ can exist in either a resting form or active form.

Similar to the $\alpha_{IIb}\beta_3$ system, the activated status of β_1 integrin is dependent on the activation state of the cell which may induced by a number of stimuli such as PMA and antibodies to the β_1 subunit. Inactive $\alpha_4\beta_1$ on peripheral B lymphocytes is converted to a high-affinity state upon brief stimulation with the phorbol ester PMA or monoclonal antibodies (Postigo, 1991). Integrins $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ in T-cells can be activated by PMA or β_1 specific monoclonal antibodies. These activated integrins bind to their respective ligands within minutes of exposure to the stimuli without an increase in receptor surface expression (Wilkins,1991; Chan,1991; Shimizu,1990;). Similarly, treatment of peripheral blood lymphocytes and cultured haematopoietic cell lines with β_1-specific monoclonal antibodies increases adhesion to fibronectin via $\alpha_4\beta_1$, $\alpha_5\beta_1$ and to laminin via $\alpha_6\beta_1$ (Kovach, 1992; Arroyo,

1992). So it is apparent that ligand and receptor activation are accompanied by conformational changes in integrins that result in increased ligand affinity. The mechanisms of affinity modulation still need to be ielucidated.

VI. THE ROLES OF INTEGRIN'S CARBOHYDRATES IN INTEGRIN FUNCTION

In general, the biological functions of many glycoproteins are not detectably different if the carbohydrates are removed (Creighton, 1993). But in a few cases, the carbohydrates are involved in the biological activity of the protein. The most relevant properties of glycosyl groups attached to the protein are 1)their variable structures, which permit specificity in their interactions with other molecules; 2) their hydrophillic nature.

There are two types of glycosylation called N-type and O-type. N-type glycosylation occurs exclusively on the nitrogen atom of Asn side chains. O-glycosylation occurs on the oxygen atoms of hydroxyl, particularly these of Ser and Thr residues (Spiro, 1973; Wagh, 1981; Kornfeld, 1976).

β_1 integrin has 778 amino acid residues. The predicted molecular weight from sequence data is 86.3-KDa. The molecular weight of β_1 expressed on the cell surface is 130-KDa under reduced conditions. Obviously β_1 integrin is highly glycosylated protein. There are twelve potential N-glycosylation sites on the molecule (Sonnenerg, 1993). In recent years some papers have reported that carbohydrates

are involved in the biological activity of integrin. Integrin-dependent cell adhesion to fibronectin is greatly promoted by optimal GM_3 concentration at the surface membrane (Zheng, 1993). Cell adhesion mediated by $\alpha_4\beta_1$ (to fibronectin) or $\alpha_6\beta_1$ (to laminin) is inhibited by modifying N-glycosylation processing of the integrin receptor (Akiyama, 1989). N-glycosylation both of the α and β_1 subunits of $\alpha_5\beta_1$ integrin is essential for association of these subunits and for optimal binding to fibronectin (Zheng, 1994). All the previous studies sugest that the carbohydrates of β_1 integrin do affect its binding capacity in vitro.

VII. RESERACH AIMS

β_1 integrns are widely expressed on lymphocytes and leucocytes and play a critical role in lymphocyte homing and in response to mediators or inflammation (Hynes, 1990; Shimizu, 1991). To identify regions involved in the biological function of β_1 integrin, a panel of β_1-specific monoclonal antibodies were generated in this laboratory and assessed for their abilities to influence lymphocyte capacity to adhere to ECM. The human T leukaemic cell line Jurkat was used in these binding assays. This cell line expresses $\alpha_2\beta_1$ integrin and displays a low spontaneous level of adherence to collagen. However, treatment of Jurkat cells with different β_1-specific antibodies can change the adherent capacity of lymphocytes. Base on the binding assay, all of the monoclonal antibodies can be divided into three categories: 1). activating antibodies which induce lymphocyte's adherence to collagen, such as B44, N29, JB1B, B3B11. 21C8). 2). inhibitory antibodies which prevent lymphocyte's adherence to collagen, such as 3S3, JB1A. 3). the neutral antibodies which do not influent lymphocyte adhesion, such as JB1. A part of binding assay results were shown in Figure (3) which were done by

Sherri Gregorash. Previous studies have demonstrated that treatment of Jurkat with α_2-specific

activating monoclonal antibody JBS2 also causes increased cellular adherence to collagen via $\alpha_2\beta_1$

integrin (Stupack et al, 1993). Similarly , a number of reports indicated that some antibodies to the

β_1 chain could activate integrin binding to extracelluar matrix (Kovach, 1992; Arroyo, 1992; Van de

Wiel-Van Kemenade, 1992). All these results suggest that β_1 mediated adhesion can be upregulated

or down regulated on lymphocytes. Mapping the regions recognized by these functional antibodies

may provide some insight into understanding the regulation of β_1 integrin. Controlling integrin

function may offer a molecular basis for a novel class of compounds that can modify cell adhesion

for therapeutic purposes.

 My major project described in this thesis was mapping the epitopes of β_1-specific activating

monoclonal antibodies as it may provide the means for better understanding the structural and

functional relationships of β_1 integrin. It will be useful for future study. To achieve this, a β_1 epitope

library which only can be used to identify continuous epitopes was constructed and screened. This

work has been performed in this laboratory successfully. Two monoclonal antibodies' epitopes had

been identified. We also identified some problems, such a reduced number of positive clones than

would be theretically predicated based on the the average size of the β_1 cDNA and the size of the

inserts. (there should be one positive clone in two thousand clones). There were several possible

explanation for these results: 1.the library may be not big enough and doesn't contain all the

antibodies' epitopes, 2. some epitopes are not expressed by the host(E.coli), 3.some antibodies may

have carbohydrates epitopes. So it was necessary to determine which antibodies could be used to

screen the library and which antibodies had carbohydrate containing epitopes. We hypothesized that

if activating or inhibiting β_1-specific monoclonal antibodies have carbohydrates or partial carbohydrates epitope, the carbohydrates of β_1 subunit may be involved in modulating the binding capacity of lymphocytes to ECM. A related project presented in this thesis was to determine whether carbohydrates of the β_1 subunit interfere β_1 mediated cell adhesion.

Fig. 3

The binding assay of Jurkat cell to collagen

Jurkat cells were pretreated with indicated anti- beta 1 monoclonal antibodies for one hour and presented to immobilized collagen. The adherence of cells was determined. The adherence of untreted cells was used as control.

Fig. 3

Effect of Antibodies to ß1 on Jurkat Binding to Collagen
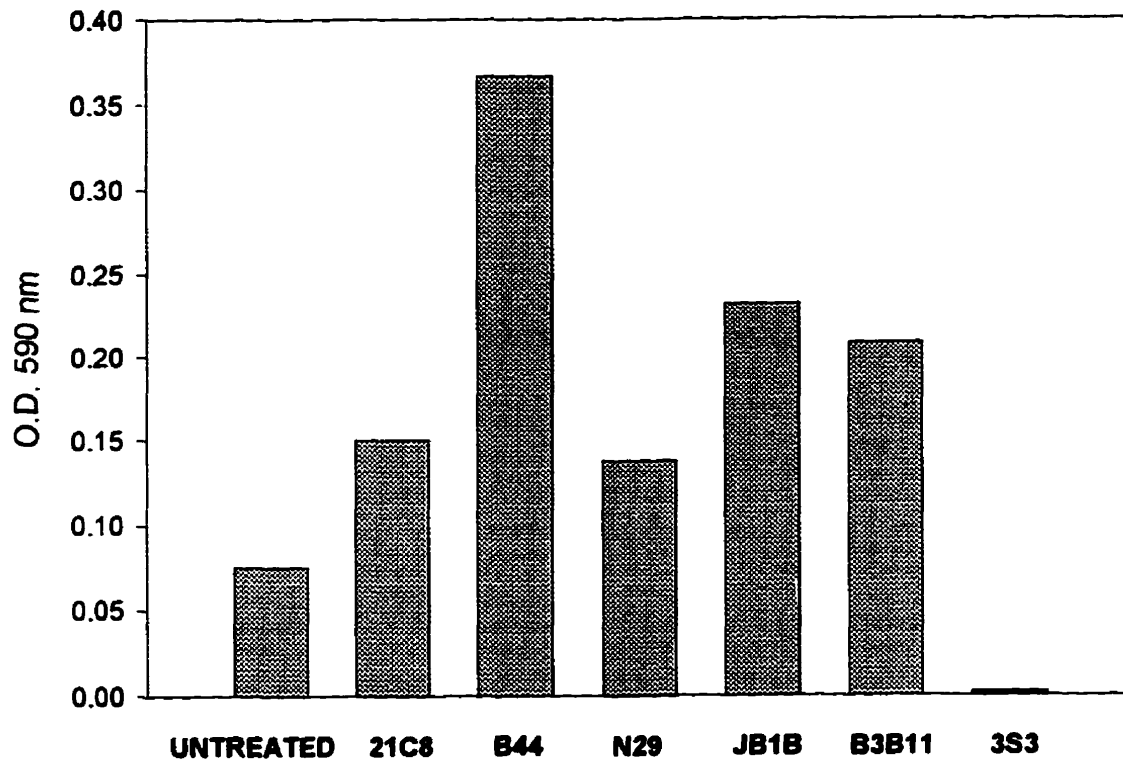
Data provided by Sheri Gregorash

MATERIALS

1. Chemicals.

Chemicals were purchased from Sigma Chemical Company(ST.Louis, MO USA), ICN Company(Costa Mesa USA) and Fisher Scientific Company(Fair Lawn, NJ USA).

2. Kits.

All the kits used for constructing the epitope library were purchased from NoVagen Company(Markham, Ontario Canada). The dsDNA cycle sequencing kit was bought from BRL(Gaitherburg, MD.USA). The plasmid purification kit was purchased from QIAGEN Inc(Chatsworth, CA). Glycofree™ deglycosylation kit was purchased from Oxford Glycosystems Ltd.(New York, USA)

3. Antibodies.

The rabbit anti-mouse IgG alkaline phosphatase conjugate for western blotting and immunoscreening was purchased from Sigma Company(ST. Louis, MD USA).The monoclonal antibodies used for immunoscreening were produced in this laboratory and listed in table (1) (Wilkins, 1996).

4. Membranes

Hybond-C, Extra, Nitrocellulose(0.45μm) used for immunoscreening was purchased from

Table. 1

Antibody properties

Antibody	Specificity	Blot[b]	Activity[c]
JB1A	β_1	+	Inhibitory
B3B11	β_1	+	Stimulatory
N29	β_1	+	Stimulatory
JB1B	β_1	+	Stimulatory
21C8	β_1	-	Stimulatory
B44	β_1	+	Stimulatory
B14-5[*]	β_1	+	Inhibitory
AIIB2[#]	β_1	+	Inhibitory
3S3	β_1	+-	Inhibitory

[b] Antibody is active in immunoblot of reduced antigen.

[c] Effect of antibody on adherence to fibronectin and collagen.

[*] B14-5, also called AP138, was a generous gift from Dr. Andrew Shaw.

[#] AIIB2 was provided by Dr. Caroline Dansky.

Amersham Company(Arlington Heights, IL.USA). Nitrocellulose membranes(0.45µm) used for western blotting were purchased from Bio-Rad(Richmond, CA, USA).

5. Radioisotopes

Adenosine 5'-[r-32P]triphosphate(ATP-r-32P), >7000Ci/mmol, was purchased from Amersham(Arlinton Heights, IL. USA).

6. Cell line

Jurkat was provided by Drs.C.Bleakley and V.Paetkau, University of Alberta. It was grown in RPMI-1640 with 10% fetal calf serum and $5x10^{-5}M$ 2-mercaptoethanol at 37°C in a 5% CO_2 environment.

7. Other reagents

Protein A beads were purchased from Sigma Chemical Company(ST.Louis, MO USA). Poly-Prep chromatography Columns were purchased from Bio-Rad(Richomond, CA,USA). Centricon Concentrators-30 were purchased from Amicon Inc (Oakville,Ontario. Canada). N-Glycosidase F was purchased from Boehringer Mannheim (Montreal, Canada).

METHODS

THE PURIFICATION OF THE pECEFnRß1 PLASMID

Bacteria carrying pECEFnRß1 plasmid were obtained from Dr E.Ruoslahli (La Jolla Cancer Research Foundation). The pECEFnRß1 plasmid contains the full length pFnRß1 gene and Amp gene. It is 6.4Kb in length. This protocol is a modification of the procedure described in Molecular Cloning a Laboratory Mannual (Sambrook, 1989).

The stock pECEFnRß1 plasmid containing bacteria were streaked on LB agar plates containing 50µg/ml ampicillin and incubated at 37°C for overnight. A single bacterial clone was transferred into 3ml of LB medium containing the 50µg/ml ampicillin in a loosely capped 15ml tube and incubated overnight at 37°C with vigorous shaking (270 rpm). The 3ml of cultured cells were transferred into 300ml of LB media containing 50µg/ml ampicillin and incubated overnight at 37°C with vigorous shaking (270 rpm). The bacterial cells were harvested by centrifugation at 4,000g for 15 min at 4°C in a Sorval GSA rotor. The supernatant was discarded and the centrifuge bottles inverted to allow all of the supernatant to drain away. The bacterial pellets were resuspended in 20ml of ice-cold solution I (50mM glucose, 25mM Tris-Cl pH 8.0, 10mM EDTA) by vigorous vortexing and 40ml of freshly prepared solution II (0.2N NaOH freshly diluted from a 10N stock, 1% SDS) was added. The contents were gently mixed by inverting and rolling the bottle rapidly five times. The sample was placed on ice for 10min. Then 30ml of ice-cold solution III(3M Potassium acetate, 5M acetic acid)

was added. The sample was mixed by inverting 5-10 times and the solution III was allowed to disperse through the viscous bacterial lysate. The sample was placed on ice for 5 min and centrifuged at 12,000g for 5min at 4°C. The supernatant was transferred to a fresh bottle. The supernatant was applied to a QIAGEN-tip 500 column pre-equilibrated with 10ml of buffer QBT. The supernatant was slowly passed through the column twice. The column was washed with 60ml of the buffer QC. The DNA was eluted with 15ml of buffer QF and precipitated by adding 0.7 volume of isopropanol, mixing well and incubating for 30 min at room temperature. The DNA was collected by centrifugation at ≥15,000g at 4°C for 15min. The DNA pellet was washed with 15ml of cold 70% ethanol, air dried for 10min and dissolved in 80μl TE buffer (10mM Tris-Cl, 1mM EDTA pH 7.5). In order to assess the yield and quality of DNA, 1μl of above products were run on a 0.8% agarose gel and compared with DNA standards in the adjacent lanes.

DNase I DIGESTION

The method is based on the observation that bovine pancreatic DNase I causes double strand cleavage of DNA in the presence of Mn^{2+} (Campbell and Jackson, 1980). The cleavage is random but the rate can be controlled by varying the enzyme concentration, temperature or incubation period, such that any size of fragments can be generated.

The following protocol used a fixed amount of DNA with increasing dilutions of DNase I to find conditions that produced the highest quantity of the desired fragments. In this case, 10μg pECEFnRß1 plasmid were used in each reaction. First the test reaction were performed to find the

appropriate enzyme:DNA ratio, and then the appropriate reactions were performed again on a larger scale to produce enough fragments for cloning. The desired fragment length was 150-300bp.

1. Titration of DNase I

The 200µl DNaseI dilution buffer was made by adding 10x DNase I buffer(0.5M Tris-HCl pH7.5, 0.5mg/ml BSA) 20µl, 10mM $MnCl_2$ 20µl, to 160µl sterile deionized water. A 1:133 dilution was prepared by adding 1µl of DNase I to 132µl of dilution buffer. and 30µl of this dilution was added to a tube containing 15µl of dilution buffer to obtain the 1:200 dilution. The same procedure was repeated three times to obtain the 1:300, 1:450, 1:600 dilutions.

2. DNase I digestion

The following reactions were set up (all components except the enzyme were added in the 0.5ml microcentrifuge tubes at room temperature).

sample	10xDNase I buffer(µl)	10xMnCl₂ (µl)	Target DNA (µl)	DNase I (µl)	DDH₂O (µl)
1	0.9	0.9	5.5	1 of 1:200	1.8
2	0.9	0.9	5.5	1 of 1:300	1.8
3	0.9	0.9	5.5	1 of 1:450	1.8
4	0.9	0.9	5.5	1 of 1:600	1.8

The reactions were started by adding the enzyme and mixing gently, incubated at room temperature(20°C) for exactly 10 min, and stopped by adding 2μl of stop buffer (100mM EDTA, 30% glycerol, and tracking dyes). A 1μl sample of each reaction was analyzed on a 2% agarose gel and compared with the DNA molecular weight markers.

FRACTIONATION OF DNA FRAGMENTS

The full reaction mixtures were separated on 2% agarose gels. Electroelution was used to recover the desired fragments from agarose gel. The band corresponding to 150-300bp fragments was excised and electroeluted by using Unidirectional Electroelutor (International Biotechanologies Inc, New Haven) following the manufacturer's instruction. Following elution, the sample was sequentially extracted with 1 volume TE-buffered phenol, 1 volume of phenol:CIAA(1:1), (CIAA=chloroform : isoamyl alcohol 24:1) and the final aqueous phase was transferred to a fresh tube. The DNA was precipitated by adding 0.1 volume of 3M sodium acetate and 2 volume of 100% ethanol and incubating on ice for 30min. The precipitated DNA was recovered by centrifugation at 12,000g for 15min. and the pellet was washed with 70% ethanol and air-dried for 30min at RT and resuspended in 30μl TE buffer(10mM Tris-HCl pH8.0, 1mM EDTA). The DNA concentration was estimated by assessing the absorbance of a 3μl sample in 697μl TE buffer at 260nm.

SINGLE dA TAILING

The Novagen single dA tailing kit was used. The kit supplies the reagents needed to blunt the DNA fragment ends and to add a single 3' dA residue in the consecutive reaction.

For blunting the DNA ends, 2.5μl(about 1-1.1μg) 150-300bp fragments, 2.5μl flush buffer(0.5M

Tris-HCl pH8.0, 50mM MgCl$_2$, 1mg/ml BSA), 2.5μl 10X dNTP mix(1mM each dCTP,dGTP, dTTP, dATP), 1.25μl 20X DTT(100mM), sterile deionized water 15.75μl, 0.5μl T$_4$ DNA polymerase(1-2units) were mixed in a microcentrifuge tube and incubated at 11°C for 20 min. The reaction was stopped by heating for10 min at 75°C.

For dA tailing,the entire flushing reaction was used. To the same microcentrifuge tube, 8.5μl 10X dA tailing buffer(100mM Tris-HCl pH 9.0, 0.5M KCl, 0.1% gelatin, 1% Triton-X-100), 51μl sterile deionized water were added and the reaction was started by adding 0.5μl Tth DNA polymerase(1.25 units) and mixing gently with the pipet tip. The sample was incubated at 70°C for 15min and then extracted with 1 volume of CIAA, vortexing vigorously for 1 min and centrifuging at 12,000g for 1min. The aqueous phase was transferred to a fresh tube and stored at -20°C.

LIGATION TO pTOPE-T-VECTOR

For a standard reaction, 50ng of pTOPE-T-Vector was ligated with different amounts of insert DNA in a final volume of 10μl. The following components were mixed in 0.5ml microcentrifuge tube: 1μl 10X ligase buffer(200mM Tris-HCl pH7.6, 50mM MgCl$_2$, 0.5μl 10mM ATP, 1μl of pTOPE-T-Vector(50ng/μl), xμl tailing products, yμl water. T4 DNA ligase 0.5μl(2-3 Weiss units) was added to start the reaction by gently stirring with a pipet tip. The samples were incubated at 16°C for overnight.

TRANSFORMATION

NovaBlue(DE3) competent cells are the host cells for transformation. Cells(200μl) were thawed on ice and aliquot of 20μl of cells were dispensed in pre-chilled eppendorf tubes. 1μl of ligation products

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were added directly to the cells and mixed gently and placed on ice for 30min. After which the tubes were heated for exactly 40 seconds at 42°C in a water bath, placed on ice for 2 min. The cells then were mixed with 80μl of room temperature SOC medium and shaken at 250 rpm at 37°C for 1 hour. 50μl of each transformation was plated on LB agar containing 50μg/ml carbenicillin plus 15μg/ml tetracycline and incubated overnight at 37°C.

IMMUNOSCREENING OF β₁ EPITOPE LIBRARY

The plates were overlaid with nitrocellulose filters. The filters were marked by poking a needle at three asymmetric places into the filter and plate. After 1 min contact with plates, the filters were peeled off and placed on the semi-wet paper towel (colony side up). The plates were reincubated at 37°C for several hours to regenerate the colonies. The papers with filters were placed in a large glass dish containing a small beaker with chloroform soaked tissues. The dish was covered tightly with Saran wrap and left at RT for 15min. The filters were removed from the chamber and placed colony side up on a piece of Whatman 3MM paper saturated with denaturing solution(20mM Tris-HCl pH7.9, 6M urea, 0.5M NaCl) for 15min at RT. The filters were blocked for 30 min with blocking solution(1% gelatin in TBST(10mM Tri-HCl pH 8.0, 159mM NaCl, 0.5% Tween-20) and washed twice with TBST with shaking and incubated with a panel of monoclonal antibodies diluted in TBST for 30-60min. The antibody solution was removed. The filters were washed three times with TBST for 10min each with shaking and incubated with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted 1:2500 in TBST for 30-60 min. The filters were washed three times as above and one time with buffer C(0.1M NaCl, 50mM MgCl₂, 0.1M Tris-HCl pH9.5). The reactive colonies were visualized by adding colour development solution[0.375mg/ml of NBT(nitroblue tetrazolium chloride)

and 0.165mg/ml BCIP(5-bromo-4-chloro-3-indolyl phosphate) in buffer C]. The colour development was stopped by rinsing the filters with water. The positive colonies were picked from the master plate, replated and rescreened until pure positive colonies were obtained. Pure positive colonies were rescreened with individual antibodies to identify which antibody reacted with these positive colonies. The filters were cut into strips and each strip reacted with a different antibody.

PLASMID PREPARATION AND SEQUENCING

Single positive colonies were transferred into 5ml of LB medium containing 50μg/ml carbenicillin and 15μl/ml tetracyclin in a culture tube and incubated at 37°C with vigorous shaking(270 rpm) for overnight. A 1.5ml sample of the culture was transferred into microcentrifuge tube and centrifuged at 12,000g for 1 min. The media were removed. The pellet was left as dry as possible, resuspended into 100μl of ice-cold solution I(50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA), mixed with 200μl of freshly made solution II(0.2N NaOH, 1% SDS), and left on ice for 3min. Then 150μl of ice-cold 3M sodium acetate pH5.2 was added and mixed by inversion and left on ice for 5min. The sample was centrifuged at 12,000g for 5 min. The clear supernatant was transferred to a fresh tube and extracted with 400μl phenol:CIAA(1:1) by vortexing for 30 seconds. The top aqueous phase was transferred to a fresh tube and mixed with 800μl ethanol by vortexing and left at RT for 10min. The sample was centrifuged at 12,000g for 15min and the supernatant was discarded. The pellet was washed with 70% ethanol, air dried, resuspended into 30μl TE buffer containing 20μg/ml RNase and incubated at 37°C for 30min. The RNA breakdown products were removed by adding 10μl of 30% PEG-8000(polyethylene glycol), 1.5M NaCl, vortexing thoroughly, incubating on ice for 60min and centrifuging at 12,000g at 4°C for 15min. The supernatant was removed and the small DNA pellet

was left behind. The pellet was rinsed successively with 100% ethanol and 70% ethanol, air dried, and finally resuspended in 20μl TE buffer. The plasmid quality was assessed by running 2μl of above products on a 1% agarose gel.

DOUBLE STRANDED-NUCLEOTIDE SEQUENCING

The forward T7 gene 10 (GAGGTTGTAGAAGTTCC) and the reverse T7 terminator (ACCGCTGAGCAATAACTAG) primers were used to sequence the inserts of the positive colonies. The Sanger dideoxy-mediated chain termination methods was used. The protocol used was described by T.Maniatis in Molecular Cloning Book(page 13.42-13.77).

EXTRACTION OF FUSION PROTEIN

A single clone was cultured in 3ml LB media containing 50μg/ml carbenicillin at 37°C at 270 rpm for overnight. 1.5 ml cultured cells were harvested by centrifugation at 12,000g for 1min, washed once with TE buffer(50mM Tris-Cl pH8.0, 2mM EDTA), resuspended in 150μl TE buffer, lysed by adding 150μl of 2x reducing sample treatment buffer(62.5mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and heating at 100°C for 5min. The sample was ready for loading.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS(SDS)

SDS-PAGE gel was used to separate proteins according to molecular weight. The discontinuous buffer system of Laemmli (1970) was used. All reagents were electrophoresis purity grade. The gels were in 0.025M Tris-Cl, 0.192M glycine, 0.1% SDS and run at 30 milliamps for two sides of minigel and 60 milliamps for two sides of large gel until the tracking dye reached the bottom of the gel.

Estimation of molecular weight was done by using the rainbow molecular weight standards(Amersham). Myosin 200-kDa, phosphorylase b 97k-Da, bovine serum albumin 69-KDa,carbonic anhydrase is 46-kDa, trypsin inhibitor 30-Kda. The gels were either coomassie blue stained to visualize protein bands or used for western blotting.

COOMASSIE BLUE STAINING

After electrophoresis, the gels were incubated with 50ml staining buffer(0.25% coomassie brilliant blue R-250 in 50% trichloroacetic acid at RT for 20min with shaking, drained and mixed with 50ml destaining buffer (50% menthanol, 10% acetical acid) several times until the bands were clearly visible.

IMMUNOBLOTTING

The proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane(NCM) by using LKB 2117 Multiphor II Electrophoresis system following the instructions provided by manufacturer. The NCM with bound protein was immersed in blocking buffer(3% BSA in 10mM Tris-HCl pH7.5, 100mM NaCl, 0.5% Tween-20) to prevent non-specific binding for 1hr at RT or overnight at 4°C. The membrane was then reacted with primary antibody solution at RT for 1hr with gentle shaking and washed three times with TBST(10mM Tris-HCl pH8.0, 150mM NaCl, 0.5%tween-20) for 10min each time, then incubated with secondary antibody(rabbit anti-mouse whole IgG alkaline phosphatase conjugate diluted 1:2500 in TBST) 1hr at RT. The NCM was washed three times as above. The fourth wash used the buffer C. The method used for colour development was

described in immunoscreening part (page32).

PREPARATION OF JURKAT CELL LYSATES

Jurkat cells were harvested by centrifugation, washed twice with PBS(137mM NaCl, 2.6mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 1mM $CaCl_2$, 1mM $MgCl_2$) and Lysed by adding 1ml lysis buffer(1%NP-40, 1mM PMSF in PBS) to 10^8 cells. The sample was incubated on ice for 20min with gently shaking. The cell debris was removed by centrifugation. The supernatants were kept at -20°C.

COUPLING ANTI-ß1 mAb JB1 TO PROTEIN A BEADS

Antibody was bound to protein A beads by using the method (coupling antibody to protein A beads) described in book - Antibodies: a laboratory mannual (Harlow,E., Lane,D., 1988)

PURIFICATION OF ß1 NATIVE PROTEIN

This protocol is a modification of the procedure described by Takada. Y.K, et al. 150ml of collected Jurkat cell lysate (from $1.5x10^{10}$ cells) were centrifuged at 5000g for 10min. The supernatant was transferred to a fresh bottle, mixed with 200µl protein A beads and rotated at 4°C for 2 hours to pre-clear the lysate. Then the beads were spun down and discarded. The supernatant was mixed with 1ml JB1-protein A beads and rotated at 4°C for overnight. The beads were spun down and washed twice with Tris buffer(0.025M Tris-Cl pH7.5, 0.15M NaCl, 0.4% NP-40, 0.1% SDS, 1mM PMSF) and added into a Poly-Prep Chromatography Column. The column was washed with 10ml tris buffer, 10ml of buffer I (50mM NaCl, 0.1% deoxycholate), 10ml of buffer II (10mM Tris-Cl pH 7.5, 0.15M

NaCl, 0.1% deoxycholate). The bound integrin was eluted with elution buffer (50mM diethylamine, 0.1% deoxycholate, 5% glycerol pH11.5). The 0.5ml eluates were collected in the eppendrof tubes with 50 μl per tube of 1M Tris-Cl pH 6.8 to neutralize the eluates. The protein concentration of each fraction was determined by assessing the absorbance at 280nm. In order to check the efficiency of elution and the purity and quantity of pure ß1 glycoprotein, 20μl of each fraction was run on a 7% SDS-PAGE gel and coomassie blue stained. The fractions which contained ß1 glycoprotein were pooled and dialysed against 2 litres of PBS at 4°C overnight. The samples were stored at 4°C.

GBINDINTHEN-DEGLYCOSYLATION OF ß1 NATIVE PROTEIN

Purified ß1 native protein was used in this experiment. For each reaction, 5μg of ß1 native protein(1mg/ml) was diluted 10-fold with 20mM phosphate, 0.5% NP-40 and heated at 100°C for 5 min. The samples were cooled down to room temperature and 2μl(0.8 unit) N-glycosidase F was added to each reaction. The samples were incubated at 37°C overnight. The reactions were stopped by adding equal volume 2X reduced sample buffer(62.5mM Tris-Cl pH6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol). The native and the N-deglycosylated ß1 samples were run in adjacent lanes in a 7% SDS-PAGE gel as described as above. The proteins were transferred to NCM. After blocking, the NCM was cut into strips(native and N-deglycosylated ß1 protein were treated as one group). Immunoblotting was performed as described as above.

CHEMICAL DEGLYCOSYLATION OF ß1 NATIVE PROTEIN

Among the principal methods which have been employed for chemical deglycosylation, the use of anhydrous trifluoromethanesulfonic acid (TFMS), has been found to be the most successful in

cleaving N- and O-linked glycans non-selectively from glycoproteins, leaving the primary structure of the protein intact. The protocol was provided by the manufacturer and involves four stages. Sample preparation, deglycosylation, neutralization of excess reagent and recovery of deglycosylated polypeptide.

Sample preparation

The ß1 native protein solution was dialysed against two litres of 0.1%(V/V) trifluoroacetic acid overnight at 4°C to remove salts, metal ions and detergents in the protein solution and concentrated to 1-2mg/ml by using Centricon-30 concentrator. An aliquot of the salt free ß1 glycoprotein 0.5ml(260µg) was transferred to the bottom of a reaction vial and dried thoroughly by lyophilization(≤50milli Torr, for 30hr).

Deglycosylation

A dry ice/ethanol cold bath was prepared. An ampoule of Reagent A(trifluoromethanesulfonic acid) was broken open and 60µl of reagent B (containing toluene) was added by using dry syringe and mixed gently. The capped vial containing the dry sample was placed into the ethanol/dry ice bath to cool down for 30 seconds. Using the syringe, 50µl of the reagent A and B mixture was added to the sample by piercing the teflon-lined septum of the vial with the syringe needle and then the reagent was allowed to run slowly down the side of the reaction vessel over 20 seconds. The needle was withdrawn and the vial was left in the bath for a further 30 seconds. The vial was placed at -20°C for 5min. The contents of the vial were checked and shaken briefly to aid melting of the contents until the contents became homogenous.

Neutralization of excess TFMS

The reaction vial was removed from the freezer and the septum was removed from inside of the cap. The vial was placed in the ethanol/dry ice bath and allowed to cool for 30 seconds. 150μl of reagent C(containing pyridine) was slowly added to the vial by allowing the reagent to flow down the side of the reaction vessel over 20 seconds. The vial was placed on dry ice for 5min and then on wet ice for a further 15min. 400μl of 0.5% ammonium bicarbonate was added to the vial and mixed briefly.

Recovery of deglycosylated polypeptide

The neutralized reaction mixture was transferred to a dialysis tube and dialysed against 4x1L of 0.5% ammonium bicarbonate buffer for over 10hr. The final polypeptide solution was concentrated into 500μl by using Centrocon-30 as above. The TFMS treated ß1 polypeptide and the native ß1 protein were run in adjacent lanes on a 7% SDS-PAGE gel as described before. The proteins were transferred to NCM and the NCM was cut into strips. Immunoblotting was performed as described as above.

RESULTS

PART A.   CONSTRUCTION AND IMMUNOSCREENING OF
THE EPITOPE LIBRARY

PURIFICATION OF pECEFnRß1 PLASMID

A total of 150µg of pECEFnRß1 was obtained from a 150ml over night bacterial culture. The purity

of the DNA preparation was determinated (Fig.4) along with a comparison of the DNA level to DNA

standards for quantitation. The final concentration of the purified plasmid DNA was estimated to be

2µg/µl.

DNase I DIGESTION OF pECEFnRß1

The results of the DNase I titration indicated that a 1:200 dilution of the enzyme gave the highest

yield of 150-300 bp fragments (Fig.5). Therefore this dilution was used in all subsequent reactions,

The 150-300bp fragments were collected by electroelution. The concentration of recovered 150-

300bp fragments was about 0.525mg/ml. Approximately 15µg DNA fragments were recovered from

60µg starting DNA.

Fig. (4)

Determination of the quality and quantity of pECEFnRß1 plasmid on agarose gel

1µl of purified pECEFnRß1 plasmid(6.4Kb) and standard DNA were electrophoresed through a 0.8% agarose gel.

Lane 1. 1Kb ladder

Lane 2. 1µl of pECEFnRß1 plasmid

Lane 3. 1µg of standard DNA

Lane 4. 1.5µg of standard DNA

Lane 5. 2µg of standard DNA

Fig. 4
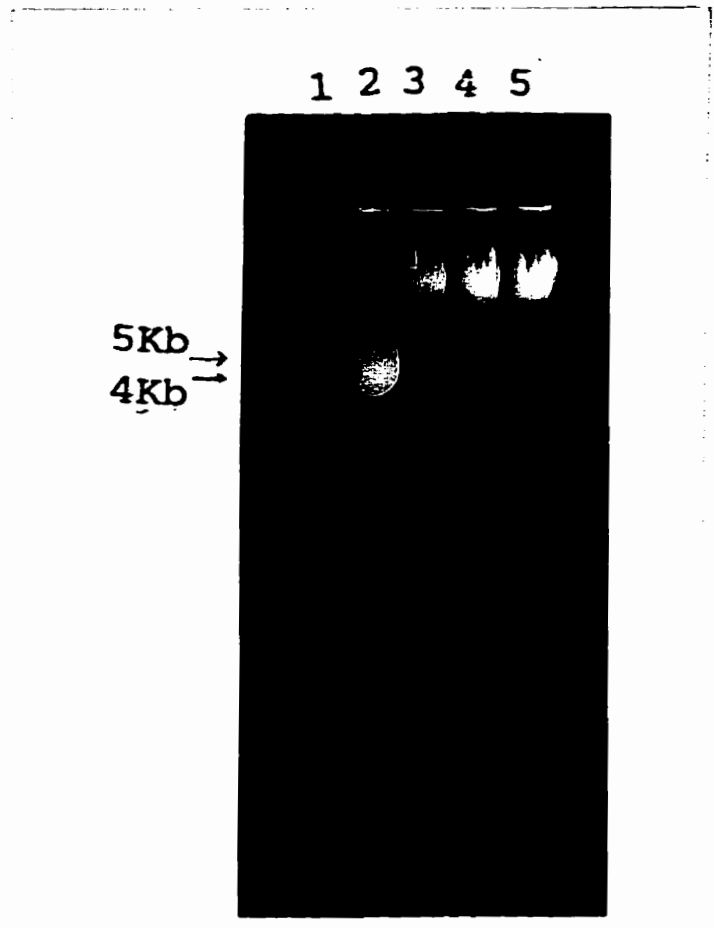
Fig.5

Determination of the quantity of the DNase I for pECEFnRß1 plasmid digestion

The amount of enzyme DNase I, required to generate the highest quantity of 150-300bp fragments

was titrated by using different amounts of enzyme to digest 10µg of pECEFnRß1 plasmid under same

condition. The products of digestion were separated on 2% agarose gel.

Lane 1. 1µl of 1:200 diluted DNase I

Lane 2. 1µl of 1:300 diluted DNase I

Lane 3. 1µl of 1:450 diluted DNase I

Lane 4. 1µl of 1:600 diluted DNase I

Fig.5

TAILING, LIGATION AND TRANSFORMATION

Transformation efficiency depends on the efficiency of tailing and ligation. The way to check the efficiency of ligation is transformation. There are several factors related to the ligation efficiency. Such as dA-tailing, pTOPE T-Vector's dT overhangs, vector:insert ratio. The tailing conditions have been optimized by the manufacturer.
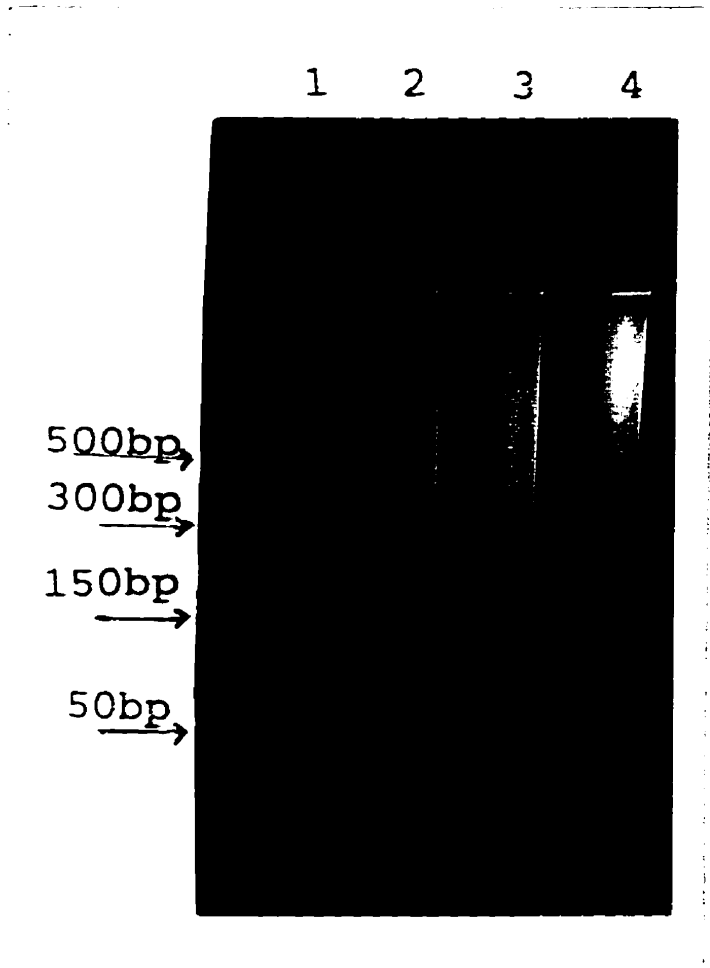
The pTOPE T- Vectors were provided by manufacturer. The vector:insert ratio is very important and usually 1:1-1:4 will yield the best results. The pTOPE T-Vector is 4000bp in length. The average size of insert is about 200bp. If 50ng vector is used in every ligation reaction, 2.5ng-10ng of insert(ratio is 1:1-1:4) should give the best result. It is necessary to titrate different vector:insert ratios to establish optimal conditions for ligation. L1(ligation 1) only contains the vector(no insert). This reaction was used to the check the percentage of pTOPE T-vector that lose the dT overhangs. L2 (ligation 2) only contains insert(no vector). This reaction was used to check whether the inserts were contaminated by vector. In L3 1.5ng of insert was used. In L4 6ng of insert was used. In L5 of 12ng insert was used. In L6 24ng of insert was used. The transformation efficiency of each reaction are shown in Table(2). It is clear that the L4 is the optimal and its vector: insert ratio is 1:2.4. L1 had 400 colonies. L4:L1=5.625. That means about 17.8% pTOPE T-Vector lost their dT-overhangs and self-ligated. L2 didn't yield any colonies. This confirms that the inserts were not contaminated by vector. Using vector:insert ratio 1:2.4, all tailed inserts were ligated into vectors to prevent the loss of the single dA and dT overhangs.

IMMUNOSCREENING

Approximately $8x10^3$-10^4 colonies of the library were screened with the monoclonal antibodies which included JB1B, B14-5, 3S3, B44, N29, AIIB2. Seven positive clones named clone A to G were picked after first screening. Clones A,B,D,E,G were positive after a second immunoscreening. Analysis of the clones with individual monoclonal antibodies indicated that the five positive clones all reacted with a stimulatory mAb JB1B. The results of clone A are shown in fig(6).

Approximately 10^4 colonies of library were screened with a second antibody pool which includes N29, 3S3 and B14-5. Five positive colonies were picked after the first screening. Only one clone was positive after the second immunoscreening. This positive clone reacted with stimulatory antibody N29.(fig 7)

Table. 2

The efficiencies of different ligation reactions

	vector:insert ratio	the number of colonies	efficiency colonies/1μg vectors
L_1	-	400	
L_2	-	0	
L_3	1:0.6	575	3.5×10^3
L_4	1:2.4	2250	37×10^3
L_5	1:5	987	11.74×10^3
L_6	1:10	1050	13×10^3

Immunoscreening of ß1 epitope library

The ß1 epitope library was immunoscreened with the first monoclonal antibody pool which includes JB1B, B14-5, 3S3, B44, N29, AIIB2. The positive clones were picked and rescreened until the pure positive clones were obtained. Then the pure positive clones were plated and screened. The filters were cut into strips to identify which mAb reacted with the positive clones.

Figure.6   shows one of the five JB1B positive clone.

A. One of the seven positive clones(named clone A) after first screening.

B. The pure clone A.

C. Clone A reacts with individual mAb of the antibody pool.

  1. pooled antibodies (positive control). 2.3S3   3. JB1B.   4.N29.

  5. Negative control.   6. B44.   7.B14-5.   8.AIIB2.

Figure.7   shows the N29 positive clone

A. One of the five positive clones(named clone C)

B. The pure clone C

C. Clone C reacts with individual mAb of the antibody pool.

  .
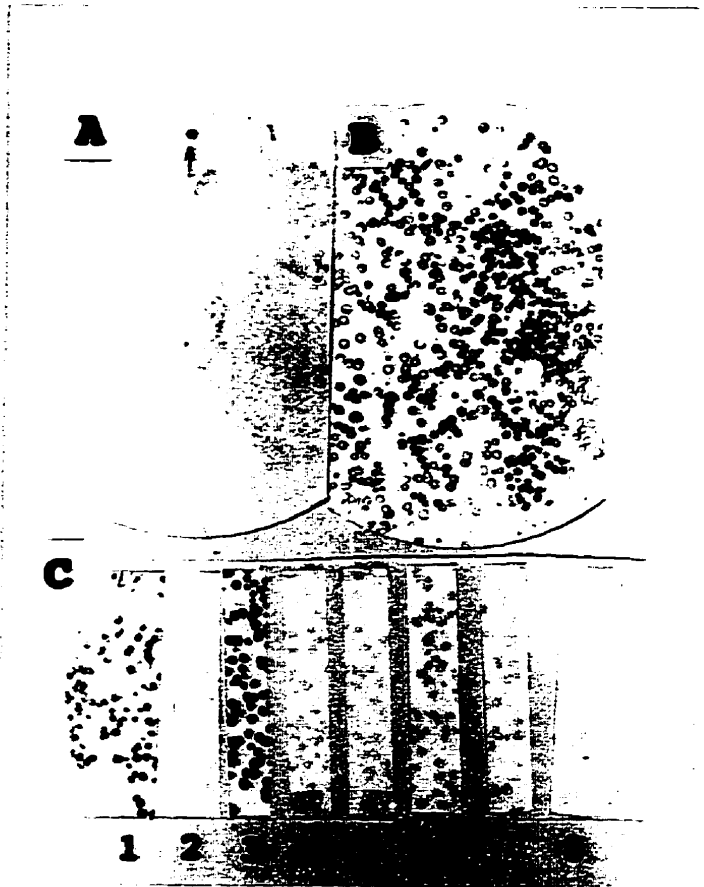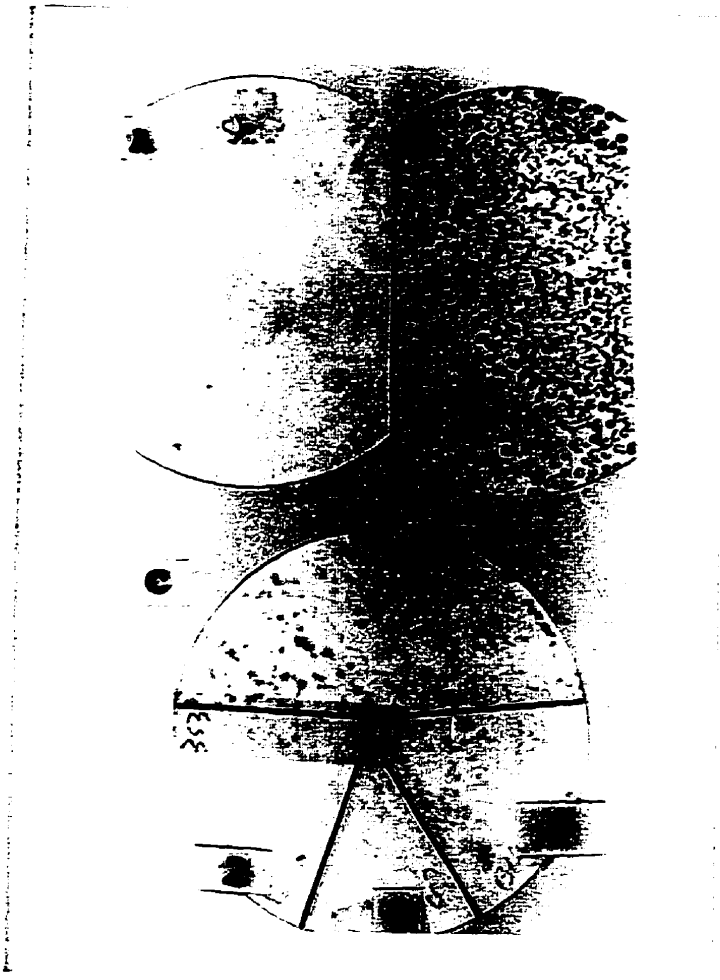 1. N29.   2.   3S3.   3.negative control.   4. B14-5.

Fig.6

Fig.7

THE SEQUENCES OF THE INSERTS OF THE mAb JB1B AND N29 POSITIVE CLONES

The plasmids of all positive clones were purified and all inserts were sequenced. The sequences of the inserts of JB1B positive clones are listed in Fig(8). Clone E contains the shortest insert which spans the ß1 sequence 647aa-705aa. This region also contains the epitope of a stimulatory monoclonal antibody B3B11 which has been identified by Dr. Wilkins , Caixia Shen and Heyu Ni. The sequence of the insert of B3B11 positive clone is listed in Fig(8).

The sequence of insert of N29 positive clone is listed in Fig (9). The insert is 163aa in length. The first 56aa of the insert is not similar to any part of ß1 sequence and contains two stop codons. But the host cell-NoVaBlue(DE3) contains the SupE44 which can insert a glutamine residue at the stop codon. The entire insert still is expressed in frame. The sequence of the remainder of insert is identical to the ß1 sequence which starts at 2aa before the mature ß1 starting site and ends at 105aa. This region also contains the epitope of an inhibitory monoclonal antibody JB1A which has been identified by Dr. Wilkins and Caixia Shen. The sequences of the inserts of the two JB1A positive clones was shown in Fig(10). Comparison of the sequences of the inserts of the N29 positive clone and JB1A positive clones were shown in Fig.(10).

Fig.8

The comparison of the inserts of JB1B and B3B11 positive clones with beta 1 amino acid sequence
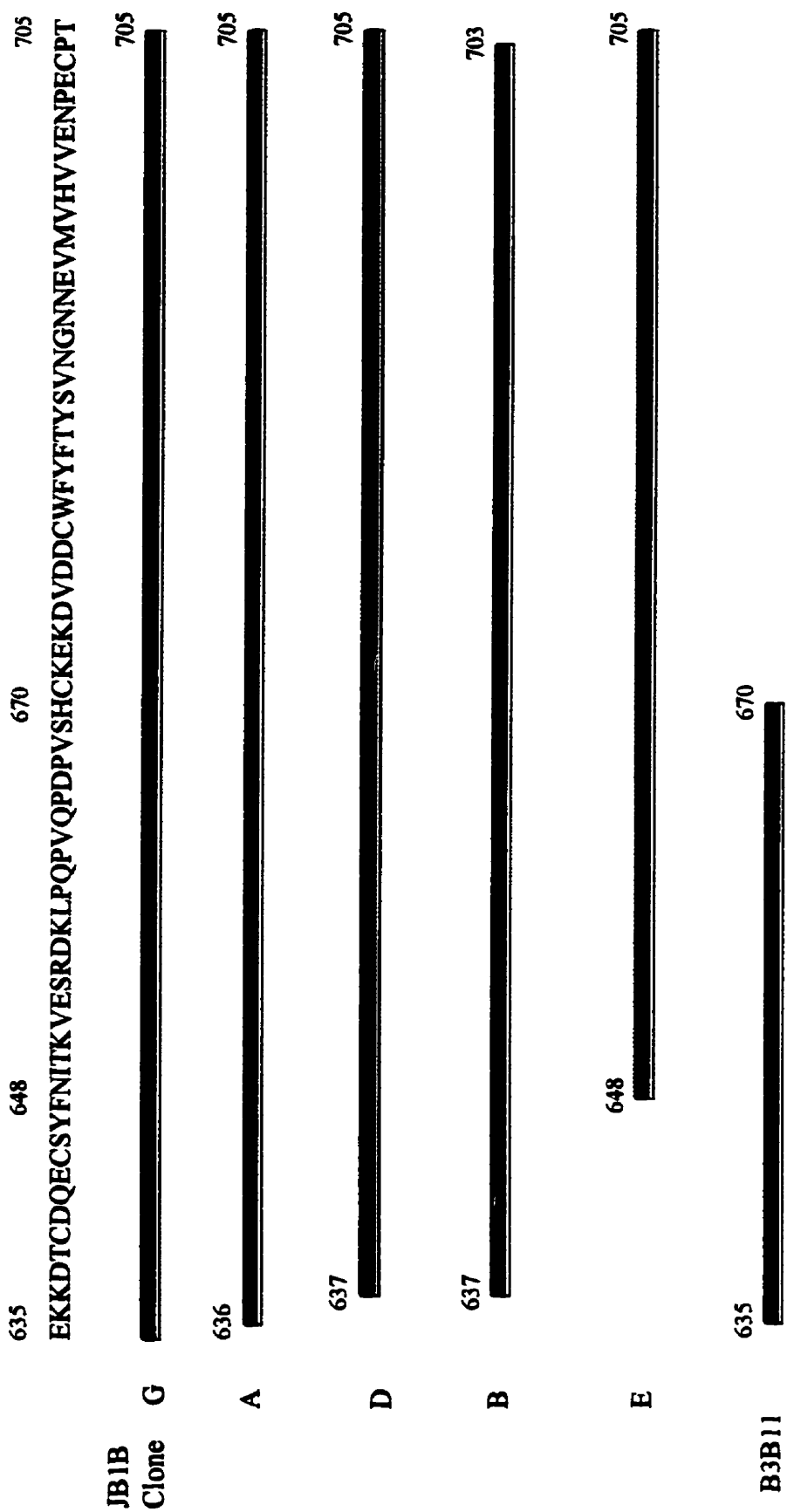
Fig. 9

The amino acid sequence of the insert of the N29 positive clone

FKYVSAHETITLINASIILKKEEYEYSTFRAPDAARKRQ*IYNQFSGLDQ*SVQFAGV

FA#QTDENRCLKANAKSCGECIQAGPNCGWCTNSTFLQEGMPTSARCDDLEALKKKG

CPPDDIENPRGSKDIKKNKNVTNRSKGTAEKLKPEDIHQIQPQQLVLRLRS

*The nucleotide sequence of these two amino acid is TGA (stop codon). NoVaBlue (DE3)

competent host cells contain the Sup E44 which can insert a glutamine residue at the codon TGA.

The starting site of mature ß1 protein

56

Fig. 10

Comparison of the insert length of N29 and JB1A positive clones

Clone N29

1   105

N   C

55   Clone A (JB1A)   93

61   Clone C (JB1A)   105

57
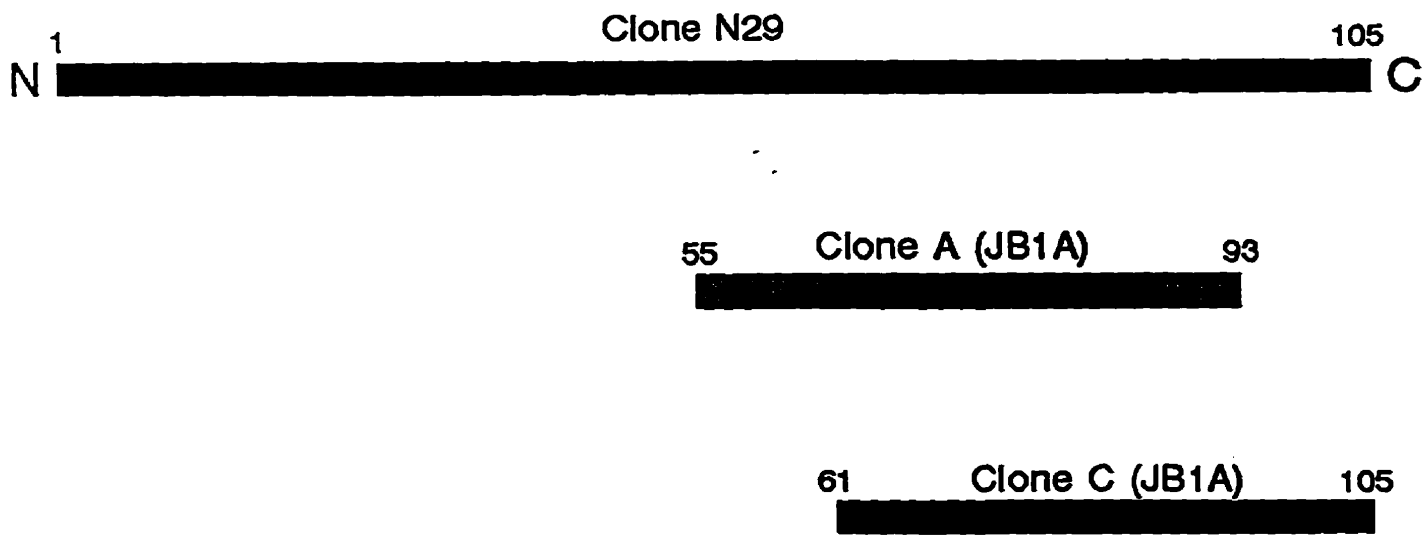
WESTERN BLOT

The fusion protein used for immunoblotting was extracted by lysing the bacteria cells in boiling 2x sample treatment buffer. Equal amounts of bacteria lysate were electrophoresed in SDS-PAGE gel under reducing conditions. The proteins were transferred to NCM. The NCM was cut into strips and each strip reacted with a different monoclonal antibody.

The fusion protein of one of five JB1B positive clones, clone E, was used in this study. Under reducing conditions, JB1B, B3B11 and 21C8 reacted with a 46-KDa band corresponding to the fusion protein. In contrast, an inhibitory anti-β_1 JB1A, failed to react with this fusion protein (fig.11B). JB1B, JB1A and 21C8 failed to react with a B3B11 positive clone (fig. 11A). These results indicated that the binding of 46-KDa fusion protein with JB1B, B3B11 and 21C8 was specific. It was apparent that 21C8 only reacted weakly with this 46-KDa fusion protein under reducing condition (data not shown). It is worth noting that 21C8 failed to react with β_1 native protein. This may suggest that the epitope of 21C8 may be a discontinuous or partial discontinuous epitope.

According to the prior studies in this laboratory, JB1B, B3B11 and 21C8 competed for binding to β_1 integrin. It appeared that these three antibodies may recognize a related region of the β_1 molecule (Wilkins, 1996). The results presented here further support this conclusion. As mentioned above, JB1B, B3B11 recognized the linear sequences of the β_1 chain since they can bind to β_1 integrin under reducing conditions. 21C8 recognized a discontinuous epitope since it is reduction sensitive.

The second positive clone obtained from screening β_1 epitope library reacted with stimulatory monoclonal antibody N29. Further immunoblot analysis of SDS-PAGE separated lysate of N29 clone, not only N29 but also a inhibitory monoclonal antibody JB1A reacted with a 51-KDa band corresponding to the fusion protein (Fig. 12A). In contrast, N29 failed to react with a 36-KDa JB1A fusion protein (from clone A) (Fig. 12B) and a 39-KDa JB1A fusion protein (from clone C) (Fig 12C). This indicated that the binding of N29 with the 51-KDa fusion protein was specific.

Fig.11

Immunoblotting the JB1B and B3B11 fusion protein with mAb JB1B, B3B11, 21C8 and JB1A

The JB1B and B3B11 fusion protein were electrophoresed on 10% SDS-PAGE gel under reduced condition. The proteins were transferred to NCM. The NCM was cut into strips. The strips were used to perform the western blot with different antibodies.

A.   The western blot results of B3B11 positive fusion protein.

B. The western blot results of JB1B positive fusion protein.

Lane 1. immunostaining with monoclonal antibody JB1B.

Lane 2. immunostaining with monoclonal antibody B3B11.

Lane 3. immunostaining with monoclonal antibody 21C8.

Lane 4. immunostaining with monoclonal antibody JB1A.

Lane 5. negative control(only incubate with the secondary antibody).

The molecular weight markers from top to bottom are 200kDa, 96kDA, 46kDa, and 30kDa respectively

Fig.11

A

1 2 3 4 5

B

1 2 3 4 5
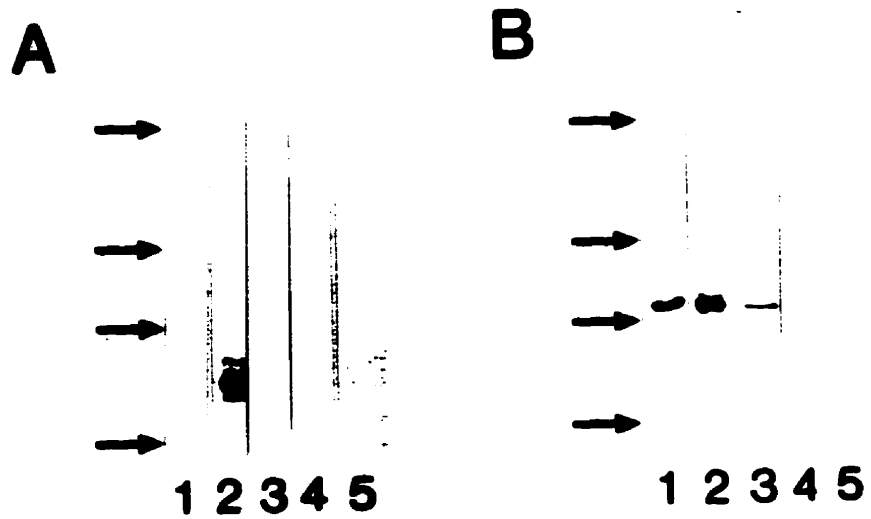
Fig.12

Immunoblotting N29 and JB1A fusion proteins with N29 and JB1A monoclonal antibody

The N29 fusion proteins were electrophoresed in 10% SDS-PAGE gel and JB1A fusion proteins were

run in 12% SDS-PAGE gel under reduced condition. The proteins were transferred to NCM. The

NCM was cut into strips and each strip was reacted with a different monoclonal antibody.

A. The western blot result of N29 fusion protein.

B. The western blot result of clone A fusion protein(JB1A positive).

C. The western blot result of clone C fusion protein (JB1A positive)

Lane 1. immunostaining with monoclonal antibody JB1A.

Lane 2. immunostaining with monoclonal antibody N29.

Lane 3. negative control(only incubate with secondary antibody).

Fig.12

A

kDa —

97→
69→

46→

30→

1　2　3

B

97kDa→
69kDa→

46kDa→

30kDa→

1　2　3

C

97kDa→
69kDa→

46kDa→

30kDa→

1　2　3
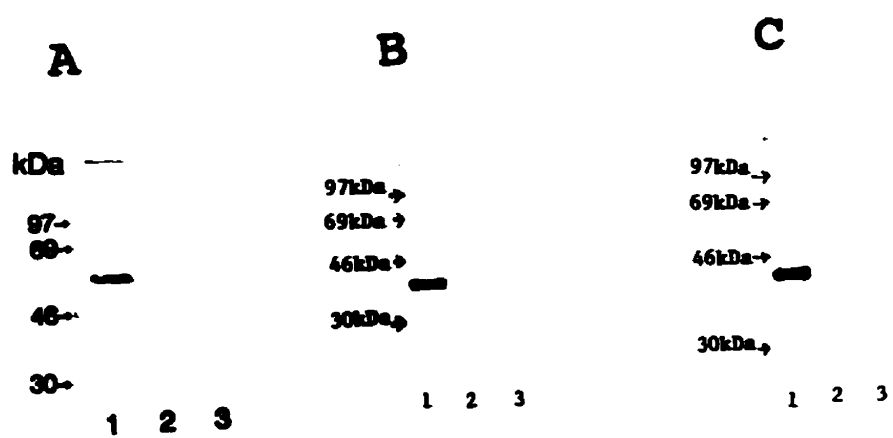
PART B.   DEGLYCOSYLATION OF ß1 GLYCOPROTEIN

Integrins are highly glycosylated proteins. In recent years, it has been reported that carbohydrates may be involved in the biological activity of integrins. We hypothesized that if activating or inhibiting β_1-specific monoclonal antibodies have carbohydrates or partial carbohydrate epitopes it suggests that the carbohydrates of the β_1 subunit may be involved in the modulating the binding capacity of lymphocytes to ECM. To test this hypothesis, purification and deglycosylation of β_1 native protein was performed.

COUPLING THE ANTI-ß1 MONOCLONAL ANTIBODY JB1 TO PROTEIN A BEADS

In order to check the efficiency of coupling, the samples saved from each step were electrophoresed on a 10% SDS-PAGE gel. After the electrophoresis, the gel was stained with coomassie blue. The results are shown in Fig.(13). The initial concentration of JB1 was about 2mg/ml. 4ml of this antibody solution were used for coupling. After absortion with 1.1 ml of protein A beads, almost all of the antibody molecules were bound to protein A beads and could not removed of by PBS. The antibody molecules were covalently linked to protein A using dimethylpimelimidate(DPM).

PURIFICATION OF ß1 NATIVE PROTEIN

The Jurkat cell lysate was made from 1.5×10^{10} cells and 1ml of JB1-protein A beads were used in each purification. In order to check the efficiency of elution, purity and quantity of purified β_1

protein, 20µl of each fraction were electrophoresed on a 7% SDS-PAGE gel under reducing conditions. The gel was stained with coomassie blue(data not shown). The protein was eluted rapidly at fraction 3 and was completely eluted with 2ml of elution buffer. About 250µg of ß1 native protein was purified from $1.5x10^{10}$ cells. Sixty litres of Jurkat cells were grown. Approximately 1mg native ß1 glycoprotein was purified from $6x10^{10}$ cells. The purified native ß1 protein was used to perform the deglycosylation.

Fig.(13)

The coupling of anti-ß1 monoclonal antibody JB1 to protein A beads

In order to check the efficiency of coupling, the samples saved from each step were electrophresed

in 10% SDS-PAGE gel. The gel was stained with coomassie blue.

Lane 1. 10µl of initial antibody solution.

Lane 2. 10µl of antibody solution after absorption with protein A beads.

Lane 3. 10µl of washing JB1-protein A beads buffer.

Lane 4. 10µl of JB1-protein A beads before linking with DPM.

Lane 5. 10µl of JB1-protein A beads after linked by DPM.

The molecular weight was given in kDa.
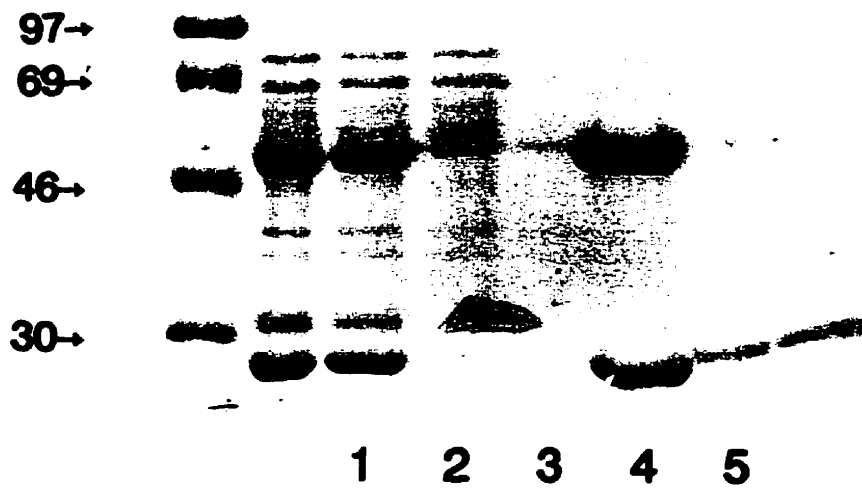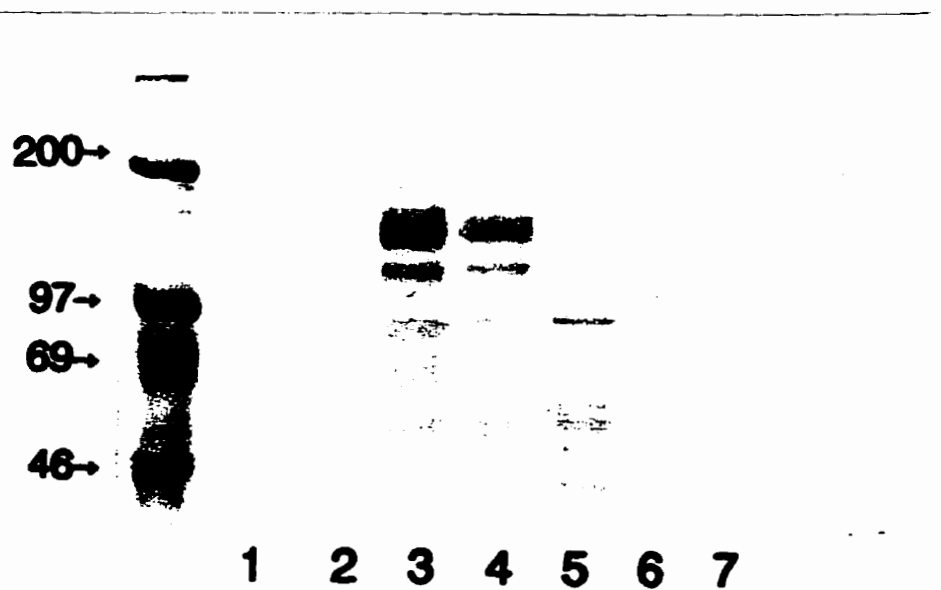
Fig.13

97→
69→
46→
30→

1 2 3 4 5

Fig.14

Determination the purity of purified β_1 native protein

20ul of each collected fractions was electrophoresed on a 7% SDS-PAGE gel under reducing condition. The gel was stained with comassive blue. Lanes 1 to lane 7 represent sequential eluted fractions. The molecular weight was given in kDa.

Fig.14

N-DEGLYCOSYLATION OF ß1 NATIVE PROTEIN

The N-deglycosylated ß1 and the native ß1 protein were electrophoresed on 7% SDS-PAGE gel under reducing conditions and the proteins were transferred to NCM. The NCM was cut into strips(the N-deglycosylated and the native ß1 protein were loaded on adjacent lanes. These two lanes were considered as one group) and each strip was reacted with monoclonal antibody D11-A, B3B11, B44, JB1B-, N29 and JB1A. The western blot results are shown in Fig(15). In each lane there are two major bands. The top band is the mature ß1 and the bottom band is the precursor ß1. The electrophoretic mobility of the N-deglycosylated ß1 is markedly increased. However the N-deglycosylated ß1 retains reactivity with D11-A, B3B11, B44, JB1B,N29,and JB1A.

TFMS DEGLYCOSYLATOPN OF ß1 NATIVE PROTEIN

The TFMS treated ß1 native polypeptide and the native ß1 protein were electrophoresed on 7% SDS-PAGE gel and the proteins were transferred to NCM. The NCM was cut into strips(the TFMS treated ß1 and the native ß1 were loaded on adjacent lanes and these two lanes were considered as one group) and each strip reacted with D11-A, N29,and B44. The western blot result is shown in the Fig (16). The TFMS deglycosylated ß1 still reacted with monoclonal antibodies D11-A, N29 and failed to react with B44. This experiment has been repeated three times and double amount of total deglycosylated ß1 was loaded in the lane which was used to react with mAb B44. It appears that stimulatory monoclonal antibody B44 recognizes an O-linked carbohydrate epitope as it reacted with N deglycosylated but not N plus O linked deglycosylated ß1 integrin.

Fig. 15

Immunoblotting analysis of N-deglycosylated ß1 protein

The native ß1 proteins(the lanes were marked as" -"in figure) and the N-deglycosylated ß1 proteins(the lanes were marked as "+" in the figure) were electrophoresed in 7% SDS-PAGE gel. The proteins were transferred to NCM. The NCM was cut into strips(the + lane and the - lane were cut as one group) and each strip reacts with different monoclonal antibodies(indicated in figure). In the each lane there are two major bands. The top band is the mature $ß_1$ and the bottom band is the precursor of $ß_1$. In the lanes reacted with JB1A and D11-A monoclonal antibody, there are lots of bands below 97kDa. They may be the degraded $ß_1$ protein.

Fig. 15
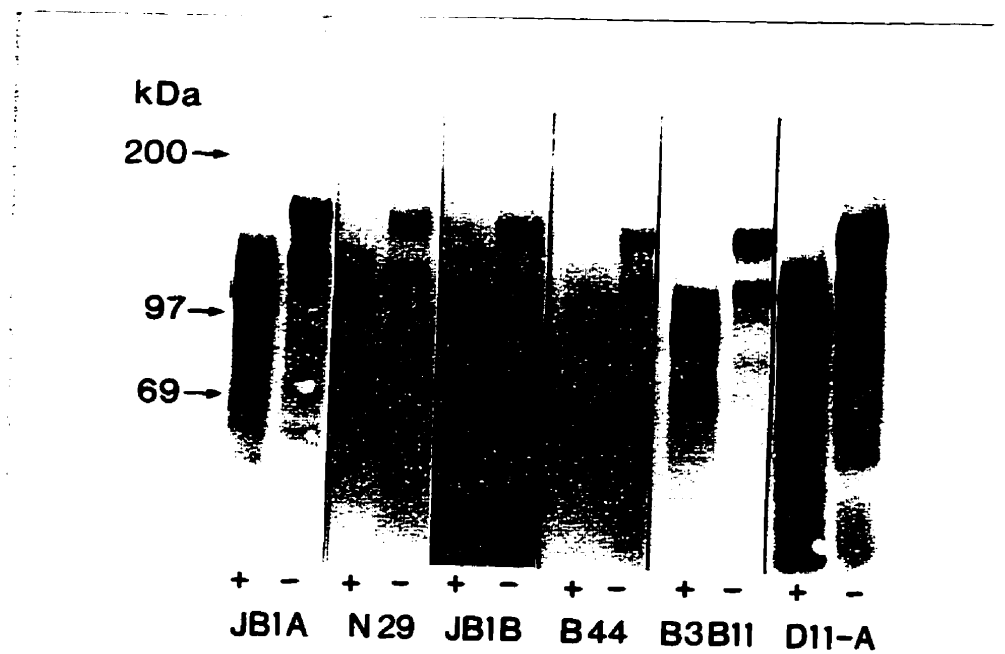
Fig. 16

Immunoblotting analysis of TFMS-treated ß1 polypeptide

The native ß1 protein(The lanes were marked as" -" in figure) and the TFMS-treated ß1 polypeptide(the lanes were marked as"+" in figure) were electrophoresed in 7% SDS-PAGE gel. The proteins were transferred to NCM. the NCM was cut into strips(+ and - lanes were cut as one group) and each strip reacted with different monoclonal antibody as indicated in the figure(16)

Fig.16
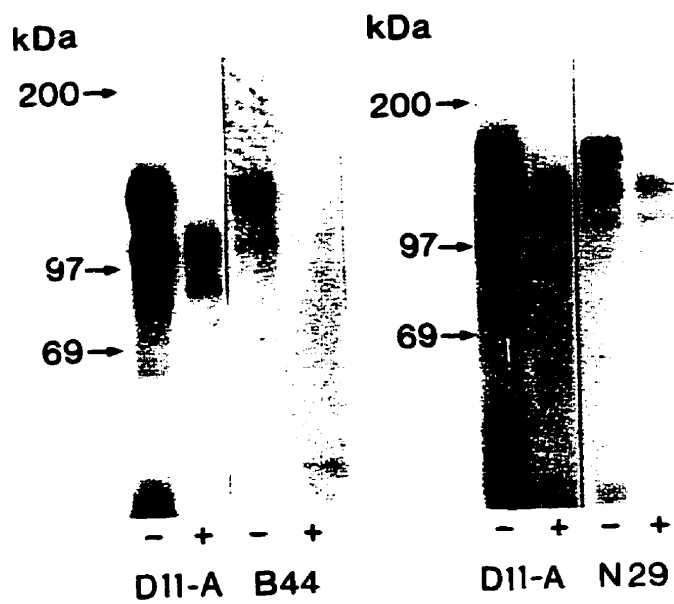
DISCUSSION

The members that form the β_1 subfamily of integrins are involved in cellular attachment to extracelluar matrix proteins and in intercellular adhesion. But the mechanism of regulation of ligand-binding capacity of integrin is still unknown. Some β_1 integrin specific monoclonal antibodies can significantly increase the ligand-binding capacity of β_1 integrin. Thus, the identification of the regions recognized by these activating monoclonal antibodies may elucidate the regulatory mechanism of the β_1 subunit at the molecular level and may be useful for potential pharmacological intervention in the the future.

The results presented in this thesis provide the following information. 1) There are no less than two distinct molecular regions influencing up-regulation of β_1 integrin mediated attachment. 2) The epitopes of a group of stimulatory monoclonal antibodies JB1B, B3B11, and 21C8 are located on the membrane proximal region (648-705aa) of β_1 integrin. 3) The epitope of stimulatory antibody N29 localizes on the amino terminal region (1-105) of β_1 integrin. 4) B44 recognizes a carbohydrate epitope which has not been localized and is sensitive to conditions which removed o-linked carbohydrates.

Through the use of a β_1 epitope library, the epitopes of JB1B and B3B11 have been localized within the region 648-705aa. The fusion protein encoded by region 648-705aa of β_1 integrin also can immunologically react with JB1B, B3B11 and under reducing condition and further confirms the

above result. Using the same approach, the epitope of B3B11 was localized within the region 636-670aa (Wilkins, 1996). The fusion protein encoded by region 636-670 of β_1 integrin only reacts with B3B11 but not JB1B . This suggest that the B3B11 epitope is in the region 648-670aa and the epitope of JB1B would appear to be contained in the region 671-705aa. The epitope of B3B11 was further localized by using three overlapping synthetic peptides which spanned the sequence of the B3B11 fusion protein. B3B11 reacts specifically with the peptide which corresponded to residues 657-670 of the β_1 sequence. JB1B and 21C8 failed to react with this peptide (Wilkins, 1996). Based on the above evidence, it is clear that the epitope of JB1B is located in the region 671-705aa on β_1 integrin.

21C8 is reduction sensitive. It does not react with native β_1 protein under reducing conditions but reacts with native β_1 protein under non-reducing conditions (data not shown). However, 21C8 did react with the 51-kDa JB1B positive fusion protein encoded by 671-705aa on β_1 integrin weakly under reducing condition. The partial epitope of the other stimulatory antibody 21C8 may be located in the region 671-705aa on β_1 integrin.

Using the same approach, the epitope recognized by another stimulatory monoclonal antibody N29 has been identified in the region of 1-105aa on β_1 integrin. The region 1-105aa also contains the epitope of a inhibitory monoclonal antibody JB1A localized by Dr.Wilkins and Caixia Shen. There are two JB1A positive clones named clone A and clone C respectively. Clone A spans AA55 to 93 on β_1 integrin. Clone C spans AA61 to 105 on β_1 integrin. The N29 positive fusion protein encoded by region 1-105aa of β_1 integrin can react with both N29 and JB1A. JB1A fusion protein (from clone A and C) only react with JB1A itself and does not react with N29. So it would appear that N29

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epitope may be located in the region corresponding to residues 1-60aa on the β_1 sequence. This was further confirmed by cloning AA1-57 into a expression vector. The expressed fusion protein specifically reacts with N29 (Heyu Ni, manuscript in preparation). But a synthesized peptide spanning amino acids 1-15 of β_1 did not react with N29 (personal communication, HY; JW). Collectively, the data suggests that the N29 epitope is located in the region from AA 1 to 57 on β_1 integrin.

There are several previous studies which have localized the epitopes identified by regulatory monoclonal antibodies to β_1 and β_3 integrins using different approaches, including interspecies chimeras, and site directed mutagenesis of the β_1 gene and β_3 genes (Takada, 1993; Shih, 1993; Du, 1995; Honda, 1995).

Shih et al have localized the epitopes recognized by a panel of stimultory and inhibitory chicken β_1-specific monoclonal antibodies by producing and expressing a series of mouse/chicken chimeric β_1 subunits (Shih,1993). These chimeric subunits were fully functional with respect to heterodimers formation, but differed in their ability to react with a panel of anti-chicken β_1 specific monoclonal antibodies. Epitopes were identified by a loss of antibody binding upon replacement of regions with the mouse β_1 subunit. They found that the epitopes for one set of inhibitory antibodies mapped toward the N-terminal region (1-260aa) of the β_1 subunit. They also found that another set of antibodies that block ligand binding, alter ligand specificity, or induce α/β subunit dissociation, mapped to the cysteine rich repeats near the transmembrane domain of the molecule and this region was considered a regulatory region. Collectively their results suggest that there are several regulatory regions on β_1 integrin. The epitopes of inhibitory monoclonal antibodies were mapped to the N-

terminal region of β_1 (1-260aa) and the epitopes of antibodies with other activities were mapped to the more membrane proximal sites.

Takada et al used a similar approach, but got different results (Takada, 1993). They have identified a small region of β_1 subunit (residue 207-218) that is critical for the binding of both human β_1 specific activating and inhibiting monoclonal antibodies by using human/mouse chimeras. This result was further confirmed by using site-directed mutagenesis. The replacement of S207N,E208K,N210E and K211V of wild type chicken β_1 resulted in the generation of the highly homologous regions of the chicken β_1 integrin which can also be recognized by some regulatory antibodies. All their data strongly indicated that the sequence 207-218 is directly involved in the binding of activating and inhibiting anti-human β_1 monoclonal antibodies to the human β_1.

In the study of functional regulation of β_3 integrin, Du reported that anti-LIBS$_2$, a β_3 specific monoclonal antibody, can stimulate fibronectin binding to the intact platelet in vitro and also stimulate fibronectin binding to purified soluble $\alpha_{IIb}\beta_3$ (Du,1991). The epitope of anti-LIBS$_2$ was localized within an 89-residue region immediately adjacent to the transmembrane domain and 400 residues carboxyl-terminal to the known ligand-binding site by using anti-LIBS$_2$ to screen a lambda gt11 expression library constructed with random fragments of β_3 cDNA. In addition, the binding of anti-LIBS$_2$ to $\alpha_{IIb}\beta_3$ was increased 20-fold by addition of a fibrinogen-mimetic synthetic peptide RGDS. Thus anti-LIBS$_2$ and ligands bind cooperatively to integrin $\alpha_{IIb}\beta_3$. There may be a functional and conformational linkage between the ligand-binding site and the antibody binding site in spit of the fact that these two binding sites are 400 amino acids apart and separated by about 16nm in the three-

dimensional structure of $\alpha_{\text{IIb}}\beta_3$ integrin. Thus, they suggested that the binding of ligand to $\alpha_{\text{IIb}}\beta_3$ integrin resulted in a conformational change which is propagated to the anti-LIBS$_2$ binding site. Conversely, the binding of the anti-LIBS$_2$ to the receptor resulted in a conformational rearrangement propagated to the ligand-binding site. Since anti-LIBS$_2$ recognizes a discontinuous epitope and there are nine cysteines within an 89aa region (602-690), they suggested that the changes of the long-range disulfide bonding pattern may be involved in the conformational change of $\alpha_{\text{IIb}}\beta_3$. All the ß subunits of integrins have fifty-six cysteines and share the same distribution pattern (Calvete,1991). The disulfide bonding pattern of the fifty-six cysteines of ß$_3$ has been partially elucidated. Cys^{655} forms a disulfide bond with cys^{406} as suggested by Calvete (Calvete,1991). Cys^{655} resides in the middle of the Pro^{602}-Pro^{690} region containing the anti-LIBS$_2$ epitope.

Honda et al (Honda,1995) have identified the epitope of an integrin ß$_3$ specific monoclonal antibody AP5 which can induce fibrinogen binding to integrin $\alpha_{\text{IIb}}\beta_3$ and also induce platelet aggregation, using recombinant human-xenopus ß$_3$ chimeras produced in a baculovirus expression system. AP$_5$ recognizes a hexapeptide sequence GPNICT (residue 1-6) at the amino terminal. The binding of AP$_5$ to $\alpha_{\text{IIb}}\beta_3$ is sensitive both to the binding of ligand and to micromolar differences in divalent cation levels. This result further confirms the hypothesis that activation related events induce global conformational changes which may be facilitated by long-range disufide bonds, and putative disulfide linkage between cys^5 and cys^{435} likely contributes to physical proximity between the AP$_5$ epitope and the C-terminal portion of ß$_3$ (Calvete,1991).

In summary, the studies of ß$_3$ integrins suggest that cys^5 and cys^{435} form the long range disulfide

bond which results in the formation of an at least 384 amino acid loop (global) structure containing two ligand-binding sites. Cys^{602} and cys^{690} form another long-range disulfide bond. These regions may be flexible and involved in conformational changes. So the binding of stimulatory monoclonal antibodies to amino terminal or carboxyl terminal regions may cause $ß_3$ integrin conformational change and expose the ligand-binding site.

In all known ß subunits of the integrin family, except $ß_4$, there are certain structural and functional features which are becoming apparent and which are highly maintained overall. These include the position and number of the fifty-six cysteines residues; the four cystine-rich repeats; the highly similar amino acid sequences within the 100-350 peptide stretch which is the ligand-binding domain for the ß subunit (Calvete,1991). Therefore many of the functional mechanisms may by shared by other integrin receptors (Hynes, 1987; Charo, 1987).

Collectively the studies in $ß_1$ and $ß_3$ integrins would seem to be most compatible with the presence of multiple regulatory regions on the $ß_1$ subunit. The stimulatory epitopes appear to be dispersed on the two termini of the $ß_1$ subunit.

The functions of integrin receptors are known to be affected by post-translational modification. In recent years, there is more and more evidence to support the hypothesis that carbohydrates of integrins may be involved in the biological activity of integrin. Early in 1990, Chammas reported that N-linked carbohydrates of $\alpha_6ß_1$ integrin play a part in the laminin-integrin $\alpha_6ß_1$ interaction by using metaperiodate oxidation, metabolic inhibition of deglycosylation and enzymatic deglycosylation

(Chammas, 1991). They found that $\alpha_6\beta_1$ integrin N-linked oligosaccharide played a part in this reaction. Binding of B16/F10 melanoma cells to laminin via $\alpha_6\beta_1$ integrin receptors was nearly abolished when cells were treated with tunicamycin, and binding of ^{125}I-labeled laminin to $\alpha^6\beta_1$ was abolished by treatment of laminin with endo-F/PNG$_{ae}$-F. This study indicated that glycosylation of both laminin and its $\alpha_6\beta_1$ integrin receptor are crucial for laminin-dependent adhesion of B16 cells. They suggested that carbohydrate moieties from each ligand interact with amino acid residues from the other, as in lectin-lectin interactions (Shoran and Lis, 1989).

The function of cell surface O-linked oligosaccharide in adhesion of HL60 cells to fibronectin has been reported (Kojima, 1994). The adhesion of the myelogenous leukaemia cell line, HL 60, to fibronectin was enhanced by blocking the elongation of O-linked oligosaccharide on the cell surface with BZαGalNAc and 12-o tetradecanoyl phorbol 13-acetate (TPA). The treatment with an N-linked oligosaccharide modifier(Swainsonine) did not affect the adhesion of HL60 cell to fibronectin. The increased adhesion of HL60 cell to fibronectin is VLA4 and VLA5 dependent and was greatly but not completely inhibited by the anti-VLA5 monoclonal antibody ,KH/33, partly inhibited by anti-VLA 4 monoclonal antibody, SG-73and completely inhibited by the combination of anti-VLA 5 monoclonal antibody and anti-VLA 4 monoclonal antibody. The expression level of VLA-4 and VLA 5 did not change during treatment with BZαGalNAc or TPA. These results strongly demonstrate that integrin-dependent adhesion of HL 60 cells to fibronectin is dependent on elaboration of cell surface O-linked oligosaccharide. These earlier studies all suggested that glycosylation of both the integrin receptor and their ligands are essential for optimal cell adhesion but the mechanism remain unknown.

The results presented in this thesis demonstrated that the O-linked carbohydrates in β_1 may play a role in β_1 integrin mediated cell adhesion. B44, a β_1 specific monoclonal antibody, can significantly enhance the adhesion of Jurket cell to fibronectin and laminin. B44 reacts with N-deglycosylated β_1 protein and fails to react with β_1 polypeptide (TFMS treated native β_1 protein) immunologically. Therefore, B44 recognizes a carbohydrate epitope or partial carbohydrate epitope on β_1 integrin and through an unknown mechanism, moderates the affinity of β_1 integrin The ligand-binding domain of the β_1 subunit may be blocked by surrounding sugar moieties. The binding of B44 to cell surface β_1 subunit may cause the conformational change of local carbohydrates and expose the ligand-binding domain. The location of the B44 epitope still needs further study.

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