Evaluation of Various Approaches to Isolate and Identify Components of Adhesion Complexes

By

Jay Krishnan

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

Master of Science

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

"Evaluation of Various Approaches to Isolate and Identify Components of Adhesion Complexes"

By

Jay Krishnan

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

of

Master of Science

To my Mom

ACKNOWLEDGEMENTS

First and foremost, I want to thank my wife and children for their support and encouragement. Without you, this chapter of my life may have turned out totally different.

To my friends and colleagues in the laboratory and in the Immunology Department, each one of you has taught me valuable lessons both in science and in life. Thank you!

This project would not have been possible without the help and co-operation of those great people in the Time of Flight laboratory, Department of Physics, University of Manitoba.

To my supervisor, Dr. Wilkins, you have taught me a lot about research and gave me chances to develop strong skills working with proteins. I will always appreciate that you took me on as a student in your laboratory.

Finally, my sincere thanks to the members of this thesis committee Drs. Werner Ens, Kent HayGlass and Peter Nickerson for their encouragement and criticism.

TABLE OF CONTENTS

ABBREVIATIONS

BSA: Bovine serum albumin

$CaCl_2$: Calcium chloride

CSK buffer: Cytoskeletal buffer

DTT: Dithiothreitol

ECM: Extra cellular matrix

FBS: Fetal bovine serum

GDP: Guanine nucleotide diphosphate

GTP: Guanine nucleotide triphosphate

HCl: Hydrochloric acid

HEPES: Hydroxyethyl piperazine ethane sulfonic acid

MES: N-morpholinoethansulfonic acid

PBS: Phosphate buffered saline

PFA: Para formaldehyde

PIPES: Piperazine ethane sulphonic acid

PLL: Poly-L-Lysine

PMSF: phenylmethanesulfonyl fluoride

SDS-PAGE: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

TBS: Tris buffered saline (25mM Tris-HCl pH 7.5 containing 150mM NaCl

TFA: Trifluoro acetic acid

uPAR: urokinase plasminogen activator receptor

VAP-1: vascular adhesion protein-1

VPM: ventral plasma membrane

ABSTRACT

Integrin mediated cellular adhesion is central to a variety of biological processes and is essential for metazoan architecture and function. Although, we have an impressive knowledge about integrins and various integrin-mediated functions, the mechanisms controlling cellular adhesion remain largely unknown. This is attributed to the lack of understanding of the many molecules that are recruited to the cytoplasmic domain of integrin at the adhesion sites, and their spatial and/or temporal sequence of interactions. This project aimed at delineating the true composition of these supramolecular complexes (adhesion complexes).

Three approaches were pursued to isolate these integrin containing supramolecular complexes intact. In the first approach, cytoplasmic domain of purified human β1 integrin was used as a bait to fish out integrin-associated molecules from fresh cell lysate. Material isolated from the ventral plasma membrane preparation of adherent cells was used as a source of adhesion complexes in the second approach. In the third approach, adhesion complexes were induced by incubating cells with anti-β1 integrin antibody coupled magnetic beads. The cell-bead pellet was magnetically separated and lysed to isolate the adhesion complexes. The molecules in the isolated complexes were resolved by SDS-PAGE and identified by mass spectrometry based proteomics.

1. INTRODUCTION

1.1. Adhesion

In 1922, biologist Warren Lewis wrote, if 'cells were to lose their stickiness for one another and for the supporting extracellular white fibers, reticuli, etc., our bodies at once disintegrate and flow off in to the ground. The so-called cement, so commonly described as existing between various types of cells, may possibly be an adhesive substance'. Now about 80 years later, we have accumulated a wealth of knowledge about cellular adhesion, the process where cells adhere to one another or to extracellular matrix. Cellular adhesion is critical to a wide variety of biological phenomena, such as fertilization, placentation, organogenesis and embryogenesis; development, tissue maintenance and wound repair; lymphocyte trafficking and inflammatory cell recruitment; and invasion and metastasis of cancer (Rojas and Ahmed, 1999; Rahman and Stratton, 1998). Adhesion is mediated by a number of molecules called adhesion molecules. There are five main structural families of adhesion molecules. They are integrins, immunoglobulin superfamily proteins, selectins, mucins and cadherins (Lodish et al, 2000; Albeda and Buck, 1990). In addition, there are several other molecules that are not easily categorized in the adhesion molecule families. For example, VAP-1 (Jalkanen and Salmi, 1993), LVAP-2 (Castro et al, 1996) and uPAR (Wei et al, 1994).

1.2. Integrins

The term integrin was proposed by Richard Hynes in 1986 (Tamkun et al, 1986) to describe a family of integral membrane receptors that promoted cellular adhesion and communication. Integrins are structurally related type I membrane-spanning glycoproteins that combine to form noncovalent αβ heterodimers. Currently 18 α and 8 β subunits have been cloned, and they may potentially form different combinations to generate 24 different receptor pairs (fig.1.1, Humphries, 2000).

Integrins can bind to a variety of ligands including extracellular matrix proteins, complement components, cell surface molecules and microorganisms (Lodish et al, 2000; Isberg and Leong, 1990; Relman et al, 1990). Integrin-ligand interactions provide physical support for cells to maintain cohesion, to permit the generation of traction forces for movement, and to organize signaling complexes to modulate cellular differentiation and fate (Humphries, 2000). The physiological relevance of integrin function is elegantly demonstrated by naturally occurring defects in the expression and/or function of these molecules as well as gene targeting experiments. The inherited bleeding disorder, Glanzmann's thrombasthenia, is caused by the loss of expression and/or function of the αIIbβ3-integrin on platelets (Perutelli and Mori, 1992; Tomiyama Y, 2000). The importance of adhesive function of β2-integrins has been demonstrated by an autosomal recessive disorder termed leukocyte adhesion deficiency. Patients with this condition have deficient expression and/or function of β2-integrin subunit. As a result, their myeloid cells fail to perform

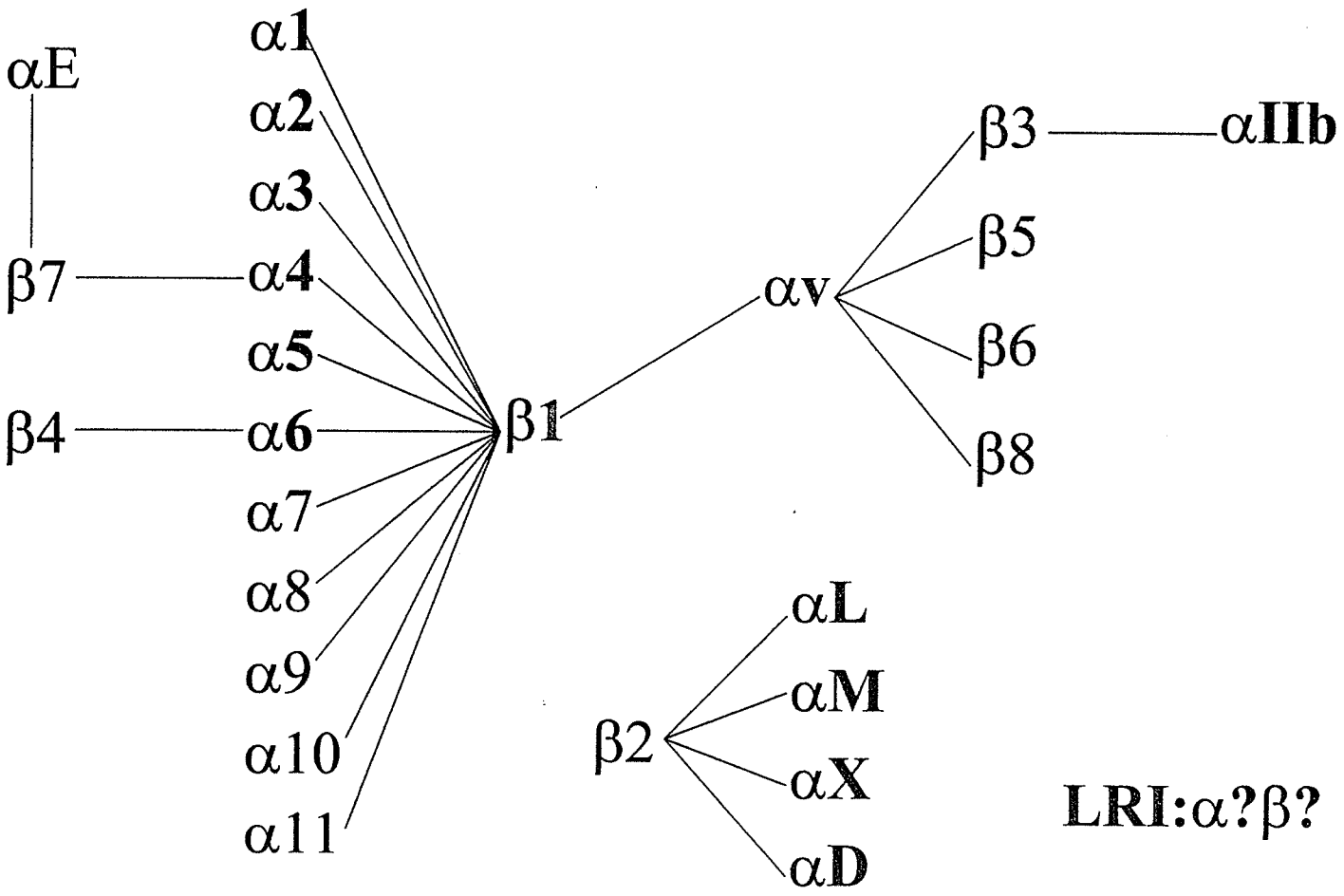

Fig. 1.1: Integrin superfamily in mammals currently comprises 25 receptors (LRI: Leukocyte response integrin)

phagocytosis and chemotaxis that leads to recurrent bacterial and fungal infections (Kishimoto et al, 1987; Fischer et al, 1988; Lipnick, 1996). Mouse embryos show lethal defects at various stages of development when β1, β4, α3, α4, α5, α6, α8 or α9 integrin is abrogated (Fässler et al, 1996). This is probably due to the deficiency of unique integrin mediated cellular responses necessary for embryonic development that are not compensated by other integrin receptors.

Several animal model studies have also shown that altered expression and/or function of integrin molecules contribute to the progression of many diseases, and therefore have implicated them as potential therapeutic targets. Anti-integrin antibodies and ligand-mimetic peptides were used to alleviate conditions such as ischemia/reperfusion injury, acute inflammation, allograft rejection during organ transplantation and various chronic inflammatory disorders (e. g., rheumatoid arthritis, inflammatory bowel disease asthma and diabetes, Curley et al, 1999; Coller, 1997; Barbadillo, 1995; Metzger, 1995).

1.3. Roles of Integrins in Regulating Immune Functions

Leukocytes express 14 integrin receptors (Hogg and Leitinger, 2001). They are essential for many aspects of immune responsiveness, such as antigen presentation, leukocyte-mediated cytotoxicity, myeloid cell phagocytosis and lymphocyte trafficking. Experiments with monoclonal antibodies to LFA-1, a β2 integrin, inhibited antigen specific cytotoxic T lymphocyte (CTL) mediated target cell killing (Gromkowski et al, 1983; Gromkowski et al, 1985). Further analysis indicated

that the interaction between LFA-1 (on CTL) and ICAM-1 (on target cell) provides the adhesive forces necessary for the two cells come together (Krensky et al, 1984; Bosco et al, 1996). In addition, Integrin also provides intracellular signals for T cell activation during antigen presentation (Damle et al, 1992; Pardi et al, 1989).

The immune system with a large but finite number of antigen-receptor-defined lymphocyte clones must respond to foreign antigen wherever it enters the body. Most of the mature lymphocytes recirculate continuously from blood to tissue and back to blood (Ford and Gowans, 1969). This disperses the immunologic repertoire, directs lymphocyte subsets to the specialized microenvironments for differentiation and survival and targets immune effector cells to sites of microbial invasion. Lymphocyte recirculation is an active mechanism of lymphocyte-endothelial cell recognition (Butcher et al, 1980) mediated by multistep sequential engagement of adhesion and signaling receptors (Butcher and Picker, 1996). Upon tissue damage and inflammation, leukocytes are recruited from the blood to the sites of injury. This process of extravasation is a critical regulatory point in the immune system, controlling the access of specialized lymphocyte subsets to particular tissues and thus influencing the nature of local immune and inflammatory responses. The successful extravasation of leukocytes out of the bloodstream across the endothelium into the underlying tissue requires the co-ordinated and sequential interaction of adhesion molecules (fig.1.2). The process of adhesion under shear flow is initiated by low-affinity binding of leukocytes to activated endothelium, which results in rolling of leukocytes along the endothelial surface. Leukocyte integrins do not function

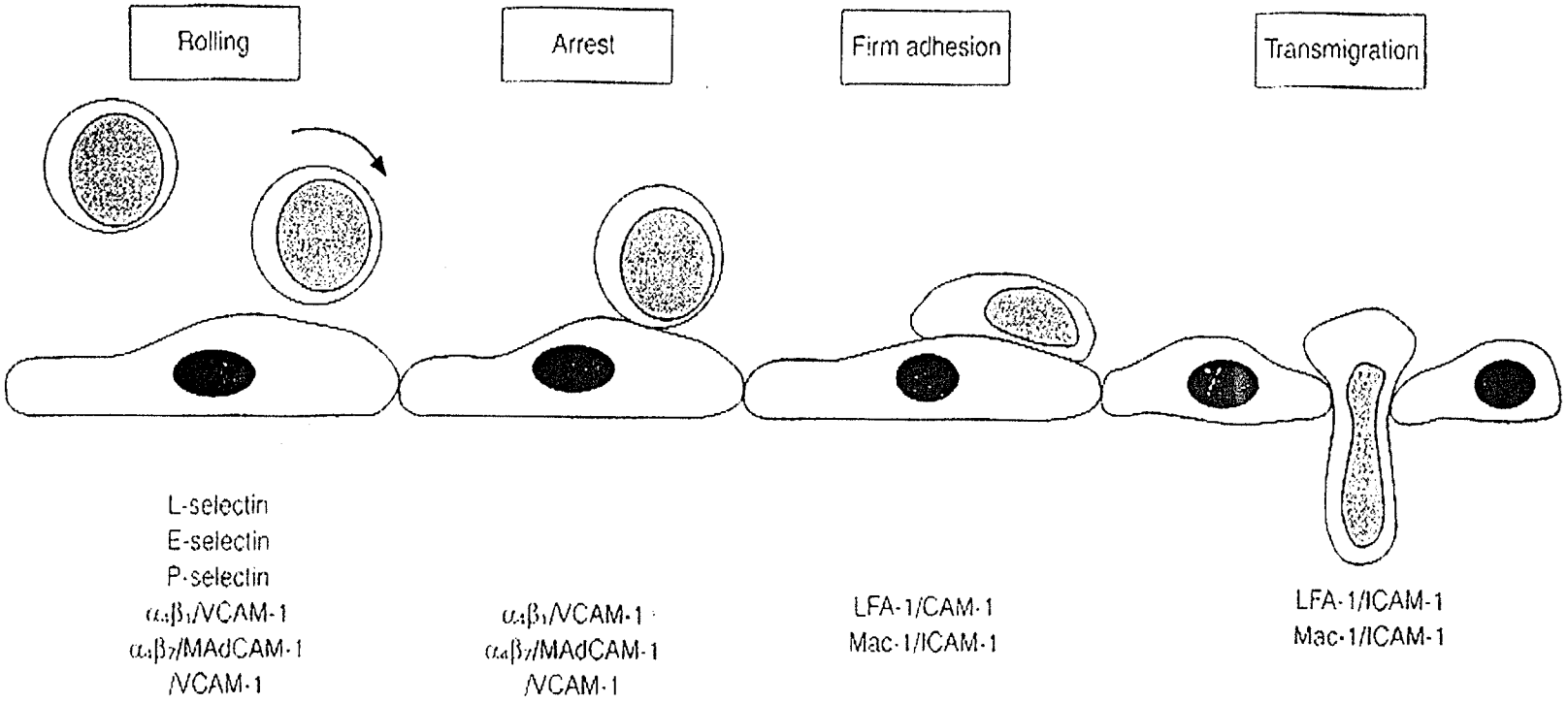

Fig. 1.2: Extravasation of leukocytes (Defilippi et al, 1997)

constitutively but undergo a process of activation. Chemokines immobilized on the endothelial surface trigger this activation. Activated integrins such as $\alpha4\beta1$, $\alpha4\beta7$, LFA-1 and Mac-1 on leukocytes convert rolling to stable shear-resistant attachment. Following tight adhesion, leukocytes migrate through the endothelium in to the underlying tissue, a process called diapedesis. Whether the migrating cell passes between endothelial cells (paracellular migration) or through the endothelial cells (paracellular migration) remains controversial (Kvietys and Sandig, 2001).

1.4. Structure of Integrin

Both α and β subunits have large extracellular and short cytoplasmic domains. The α subunits are approximately 1050 amino acids long with most of the sequences extracellular with a 15-50 aminoacid cytoplasmic domain. α chains are classified into two groups (fig. 1.3). Members of the first group are cleaved into heavy and light chains that are held together by a disulfide bridge. The second group members are single chain molecules containing an extra sequence of 180-200 residues called the I (inserted or interactive) domain (Hemler, 1990). Both groups contain multiple repeats of a sequence homologous to EF hand motif (Kawasaki and Kretsinger, 1995) that are able to bind to cations. Cation binding sites are also present in the I domain (Michishita et al, 1993) and cations are important in regulating integrin-ligand binding (Gailit and Ruoslahti, 1998).

The β subunits are approximately 780 aminoacids long with 700-730 residues exposed at the extracellular surface and 40-50 residues cytoplasmic domain (fig 1.3).

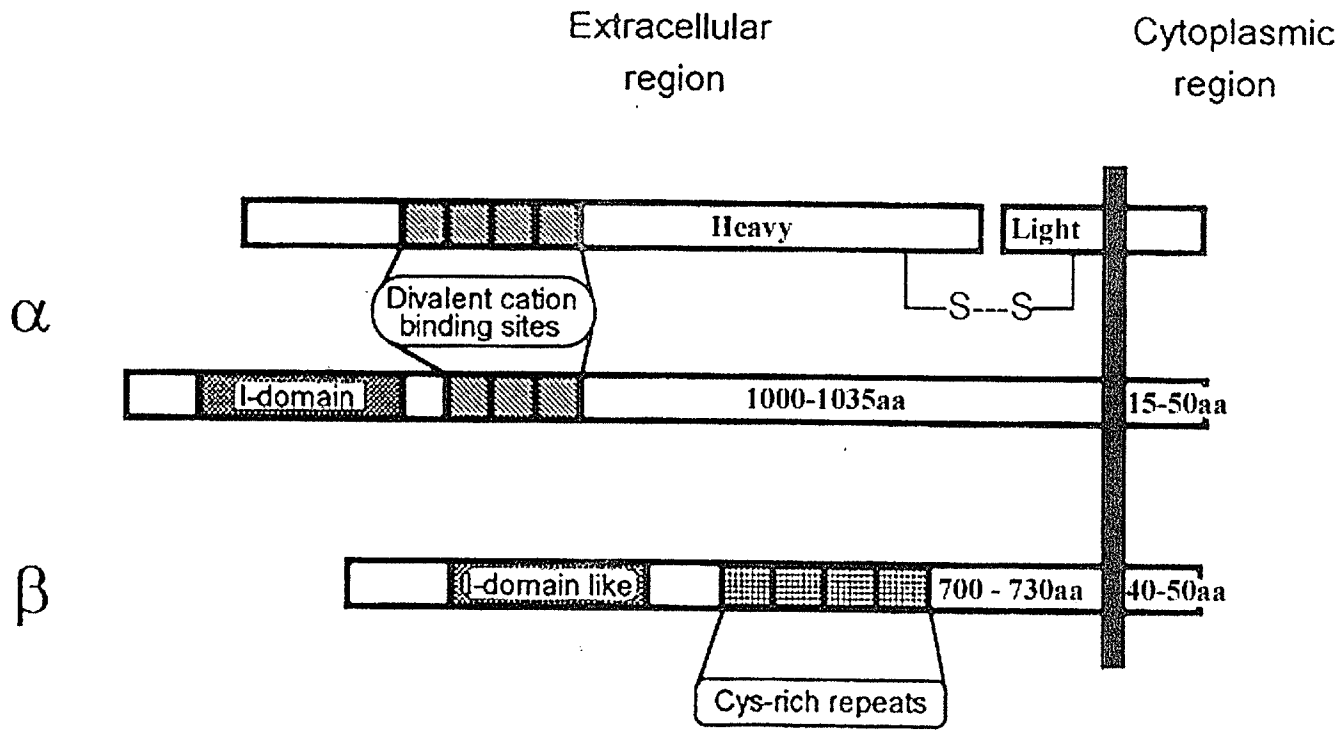

Fig. 1.3: Structure of integrin. (Defilippi et al, 1997)

β4 is an exception in being 1752 residues long and 1000 of which makes the cytoplasmic domain (Suzuki and Naitoh, 1990; Hogervorst et al, 1990; Tamura et al, 1990). Most β subunits contain 56 cysteines, whose positions are highly conserved among different β chains from *Drosophila* to human molecules (Hemler, 1990). These residues form extensive disulfide bridging leading to a highly packed three-dimensional structure that is responsible for the high resistance to protease attack (Tarone et al, 1982; Giancotti et al, 1985). Sequence analysis revealed the presence of a region homologous to the I domain of the α subunit and involved in ligand binding (Smith and Cheresh, 1990; D'Souza et al, 1990; Loftus et al, 1990; Lee et al, 1995).

The complexity of the integrin superfamily is further increased by the presence of different isoforms. There are four β1 isoforms, they are A (Argraves et al, 1987), B (Altruda et al, 1990), C and D (Languino and Ruoslahti, 1992; Zhidkova et al, 1995; van der Flier et al, 1995; Belkin et al, 1996) produced due to alternative splicing or alternative exon usage. Only the carboxy-terminal region is unique to each of these isoforms and the rest of the molecules are identical. β1A is the ubiquitous integrin β1 subunit. Different β1 isoforms has distinct and specific functions. For example, β1B has anti-adhesive property, it does not activate signaling through Fak^{125} nor does it organize focal adhesion (Balzac et al, 1993). When co-expressed with β1A or β3, β1B has a dominant negative action and inhibits cell spreading, migration, assembly of fibronectin matrix and formation of focal adhesions (Balzac et al, 1994; Retta et al, 1998). Existence of isoforms in β3 (van Kuppevelt et al, 1989), β4

(Hogervorst, et, al, 1990; Tamura et, al, 1990; Clarke et, al, 1994), α3 (Tamura et al, 1991), α6 (Cooper et al, 1991; Hogervorst et al, 1991) and α7 (Collo et al, 1993; Ziober et al, 1993) subunits have also been documented.

Several different integrins have been shown to bind to the same ligand. Eight different fibronectin receptors have been described and seven different integrins can bind to laminin. This indicates that evolution has provided a compensatory system. For example, cells lacking α5β1 fibronectin receptor still assemble fibronectin matrix *in vitro* utilizing other β1 (Yang et al, 1993) or β3 (Wennenberg et al, 1996) integrins. In several cases, distinct receptors elicit different cellular responses upon binding to a given matrix protein implying not all receptors suitable for rescue function. For example, αvβ1 and α5β1 can both bind to fibronectin, but differ in their ability to promote fibronectin matrix assembly (Zhang et al, 1993) whereas αvβ3 and αvβ5 bind to vitronectin but have different roles in cell migration (Klemke et al, 1994; Liaw et al, 1995). Contrary to a ligand being bound by a number of integrin receptors, a given receptor is able to bind to a multiple number of ligands as well. For instance, αvβ3 can bind to 10 different ligands including vitronectin, fibronectin and several others. Thus, receptors such as αvβ3 are suitable for rescue function, i.e., rescuing a cell function that would have otherwise affected by the lack of another integrin receptor.

Affinity labeling and crosslinking experiments with soluble peptide-ligands indicated both α and β subunits are required to form the ligand binding pockets (Smith and Cheresh, 1990; D'Souza et al, 1990). The first three cation-binding

domains of the α subunit and an amino terminal region between 109-119 of the β were identified as the region making contact with the ligand. Charo et al (1991) identified a region comprising of residues 204-229 of β3 contributing to ligand binding in addition to residues 109-119.

1.5. Integrin Signal Transduction

The ability of integrins to switch between inactive and active states has important physiological implications and is considered as a general property of integrin complexes (Faull et al, 1993; Altieri and Edgington, 1998; Crowe et al, 1994). Integrin activation must facilitate signal transduction for meaningful changes in cellular function. There are two major signal transduction modes described for integrin (Humphries, 2000). First, ligands or antibodies cluster integrin receptors close together and thereby trigger post-translational modifications and/or effector molecule engagement. Second, ligands, antibodies or cations induce conformational changes to integrin that create effector binding sites and/or exposure of sites for modifying enzymes. Clustering is considered to increase the avidity of molecular interactions. Clustering induced integrin activation and functions have been well documented (Stewart et al, 1996; Stewart and Hogg, 1996). Thus, the adhesion of cells to multivalent ligands in the extracellular matrix or on other cell surfaces causes accumulation of signaling complexes on the cytoplasmic face of the plasma membrane (Humphries, 2000).

Integrins, being metalloproteins, require divalent cations. In general, Mg^{++} and Mn^{++} promote ligand binding while Ca^{++} is considered as an inhibitor (Humphries, 2000; Gailit and Ruoslahti, 1988; Mould et al, 1998). These studies imply that the extracellular domain of integrins undergo conformational changes that regulate ligand binding. Studies with monoclonal antibodies to different integrin epitopes also supported this. While a number of antibodies were found to inhibit integrin ligand binding, several of them have shown to stimulate ligand binding (Ni and Wilkins, 1998; Keizer et al, 1988; Neugebauer and Reichardt, 1991; Frelinger et al, 1991; Kovac et al, 1992; Arroyo et al, 1993; Lenter et al, 1993; Mould et al, 1995).

Intracellular stimuli can also affect the state of integrin activation by acting at the cytoplasmic side. αIIbβ3 integrin becomes competent for fibrinogen binding only when platelets are activated by blood clotting stimuli (Bennet and Vilaire, 1979). Several stimuli can regulate this property, for example, thrombin activates the fibrinogen receptor on platelets and antigen receptor stimulation regulates β2 integrin-ligand binding on lymphocytes. Thus, in addition to serving as a *molecular glue* (Etzioni, 2000), integrins mediate signal transduction in both directions. Binding of ligands transmits signals in to the cell and results in cytoskeletal re-organization, gene expression and cellular differentiation (outside-in signaling, Smyth et al, 1993; Fox, 1994; Chen et al, 1994). On the otherhand, signals from within the cell can also propagate through integrins and regulate integrin ligand binding affinity and cell adhesion (inside-out signaling; Hynes, 1992; Schwartz et al 1995; Ginsberg et al, 1992). These pathways are directed to two main functions: organization of the

cytoskeleton and regulation of cellular behavior including differentiation and growth. Organization of actin or intermediate cytoskeleton during cell adhesion requires intracellular signals to trigger polymerization of cytoskeletal proteins in response to cell matrix adhesion. In addition, the ability of cell matrix interaction to control cell proliferation (Folkman and Moscona 1978) and differentiation (Menko and Boettiger, 1987; Solursh et al, 1984; Edgar et al, 1984; Dedhar, 1989) strongly implied the existence of matrix dependent signaling events. It has subsequently been shown that more upstream events, such as elevation of intracellular pH (Schwartz et al, 1989), Ca^{++} transients (Pardi et al, 1989) and protein tyrosine phosphorylation (Ferrel and Martin, 1989) can be triggered by integrins. The list of integrin-mediated signaling now comprises most of the known pathways. These include Ras/MAPK activation (Chen et al, 1994; Zhu and Assoian, 1995; Morino et al, 1995), activation of protein kinase C (Breuer and Wagener, 1989; McNamee et al, 1993) and activation of tyrosine kinases (Ferrel and Martin, 1989; Nakamura and Yakumura, 1989; Golden et al, 1990).

Although many different integrin-dependent signaling pathways are known, the molecular mechanisms by which integrins trigger these events are poorly understood. The cytoplasmic domain of integrins does not have any enzymatic activity. Thus, interaction with transducing proteins is required to start a signaling event. Studies have identified a number of intracellular proteins that associate with or are functionally coupled to integrins via these cytoplasmic tails. These proteins, integrin associated proteins, regulate and mediate integrin-induced functions. The

cytoplasmic domains of integrins thus play a pivotal role in these bi-directional signaling processes. Intensive efforts have focused on identifying cellular proteins that can directly interact with integrin cytoplasmic domains in order to elucidate molecular mechanisms by which integrin mediate bi-directional signal transduction (Dedhar and Hannigan, 1996; Hemler, 1998; Hughes and Pfaff, 1998). A complete understanding of the molecular basis of integrin regulation will require identification of these integrin-binding proteins and characterization of their activities. At least 21 proteins are known to bind to one or more integrin β tails (fig. 1.4). These proteins were discovered using various techniques such as Yeast two-hybrid screen, co-immunoprecipitation, synthetic/recombinant peptide studies, binding to purified integrin, static light scattering and equilibrium gel filtration (Liu et al, 2000). In adherent cells, these molecules and integrin receptors co-localize to form supramolecular complexes called focal adhesion complexes (fig. 1.5). Focal adhesions are specialized sites of cell attachment to the extracellular matrix where integrin receptors link the ECM to the actin cytoskeleton (Sastry and Burridge, 2000).

1.6. Cytoskeleton

Cells adopt a variety of shapes and carry out movements with the help of a complex network of protein filaments in the cytoplasm called cytoskeleton (Bannikov et al, 1982; Isenberg et al, 1979; Wright et al, 1988; Gordon and Staley, 1990). This structure acts both as muscle and as skeleton, for movement and stability. It is highly dynamic and reorganizes continuously as the cell changes shape, divides and

Binding partner	Integrin tail	Detection
Actin-binding protein		
Talin	β_{1A},β_{1D},β_2,β_3	COIP, PEP, EQ, INT, SLS
Filamin	β_{1A},β_2,β_3,β_7	COIP, PEP, 2HYB, SLS
α-actinin	β_{1A},β_2	PEP, INT, COIP, SLS
F-actin	α_2	PEP
Myosin	β_3	PEP, COIP
Skelemin	β_1,β_3	2HYB, PEP
Signaling protein		
ILK	β_1,β_3	2HYB, COIP
FAK	β_1,β_2,β_3	PEP, COIP
Cytohesin-1	β_2	2HYB, COIP, PEP
Cytohesin-3	β_2	2HYB
Other protein		
Paxillin	β_1,β_3,α_4	PEP, COIP
Grb2	β_3	PEP
Shc	β_3	PEP
β3-endonexin	β_3	2HYB, INT, PEP
TAP-20	β_5	PEP
CIB	α_{IIb}	2HYB, PEP, COIP
Calreticulin	α	PEP, COIP
Caveolin-1	α	COIP
Rack1	β_1,β_2,β_5	2HYB, PEP, COIP
WAIT-1	β_7	2HYB, PEP
JAB1	β_2	2HYB, PEP, COIP
Melusin	β_{1A},β_{1B},β_{1D}	2HYB, INT
MIBP	β_{1A},β_{1D}	2HYB, PEP, COIP
ICAP-1	β_{1A}	2HYB, PEP, INT
CD98	β_{1A},β_3	PEP
DRAL/FHL2	α_{3A},α_{3B},α_{7A},β	2HYB, PEP

Fig 1.4: Molecules that are shown to bind to the cytoplasmic domain of integrin β subunit (Liu et al, 2000). COIP: coimmunoprecipitation, PEP: synthetic/recombinant peptide studies, 2HYB: yeast two hybrid screen, INT: binding to purified integrin, SLS: static light scattering, EQ: equilibrium gel filtration

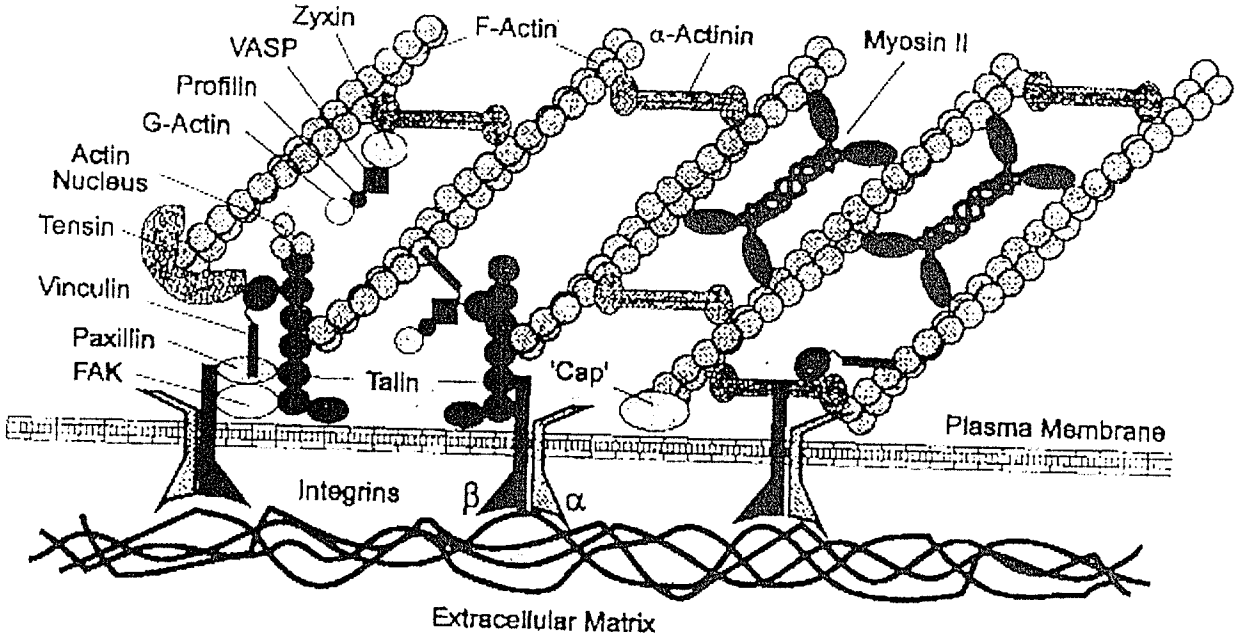

Fig. 1.5: Focal adhesion complexes (Humphries, 1997). These supramolecular complexes are formed from integrins and associated molecules

responds to its environment. Actin filaments, microtubules and intermediate filaments are the three types of protein filaments of the cytoskeleton.

The actin filaments are polymers of tightly oriented subunits known as globular (G) actin. Actin is the most abundant protein in many cells, often as much as 5% or more of the total cellular protein. Fluorochrome labeled phalloidin is commonly used to stain and visualize actin filaments in cells. Phalloidin is a toxin from *Amanita* mushroom that binds tightly along the sides of the actin filaments (Lengsfeld et al, 1974; Dancker et al, 1975; fig. 1.6). Dynamic actin filaments are a common feature of cells in motion and in the process of changing shapes. Leading edge of a migrating fibroblast extends thin sheet like processes called lamellipodia or stiff protrusions called microspikes. Longer forms of microspikes called filopodia that are most studied in developing neurons (Bernstein and Bernstein, 1977) are also formed in many motile cell types (Buehler, 1976; Praeger, 1986). All these structures contain bundles of actin filaments and are generated by local actin polymerization at the plasma membrane and they rapidly push out the plasma membrane (fig. 1.7). The cortical actin filament network lies just below the plasma membrane and provides the cell with mechanical strength.

Cortical actin may also be organized as stress fibers where actin filaments associate with motor protein myosin II and thus acquire contractile property. One end of the stress fibers is inserted in to the plasma membrane at special sites called focal contacts (fig. 1.8). The other ends may be inserted to another focal contacts or in to the meshwork of cytoskeleton inside the cell. To pull on the extracellular matrix

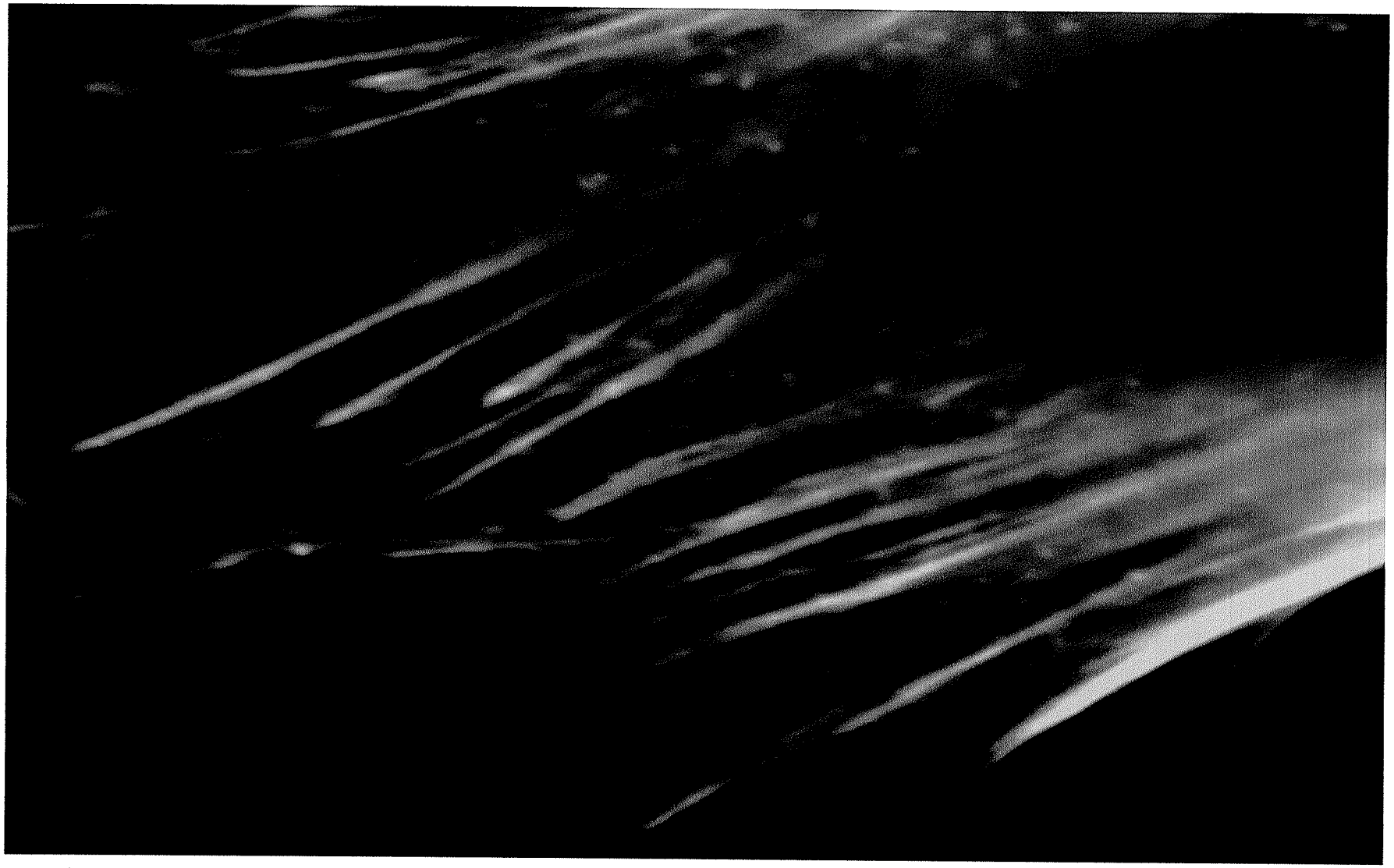

Fig. 1.6: Phalloidin-FITC stained actin filaments. Fixed and permiabilised adherent fibroblast cell was incubated with phalloidin-FITC

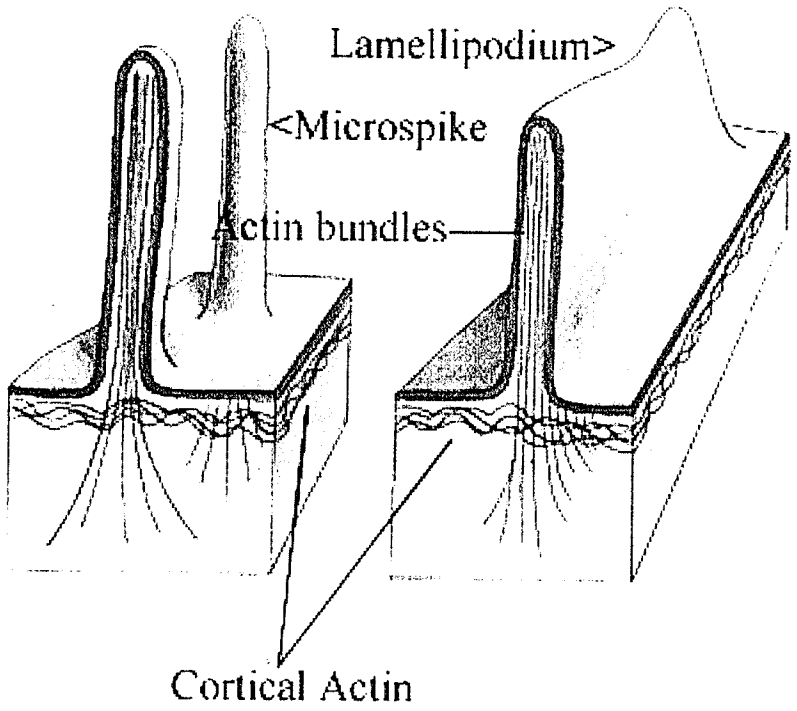

Fig. 1.7: Diagrammatic representation of actin containing structures inside the cell (Alberts et al, 1994)

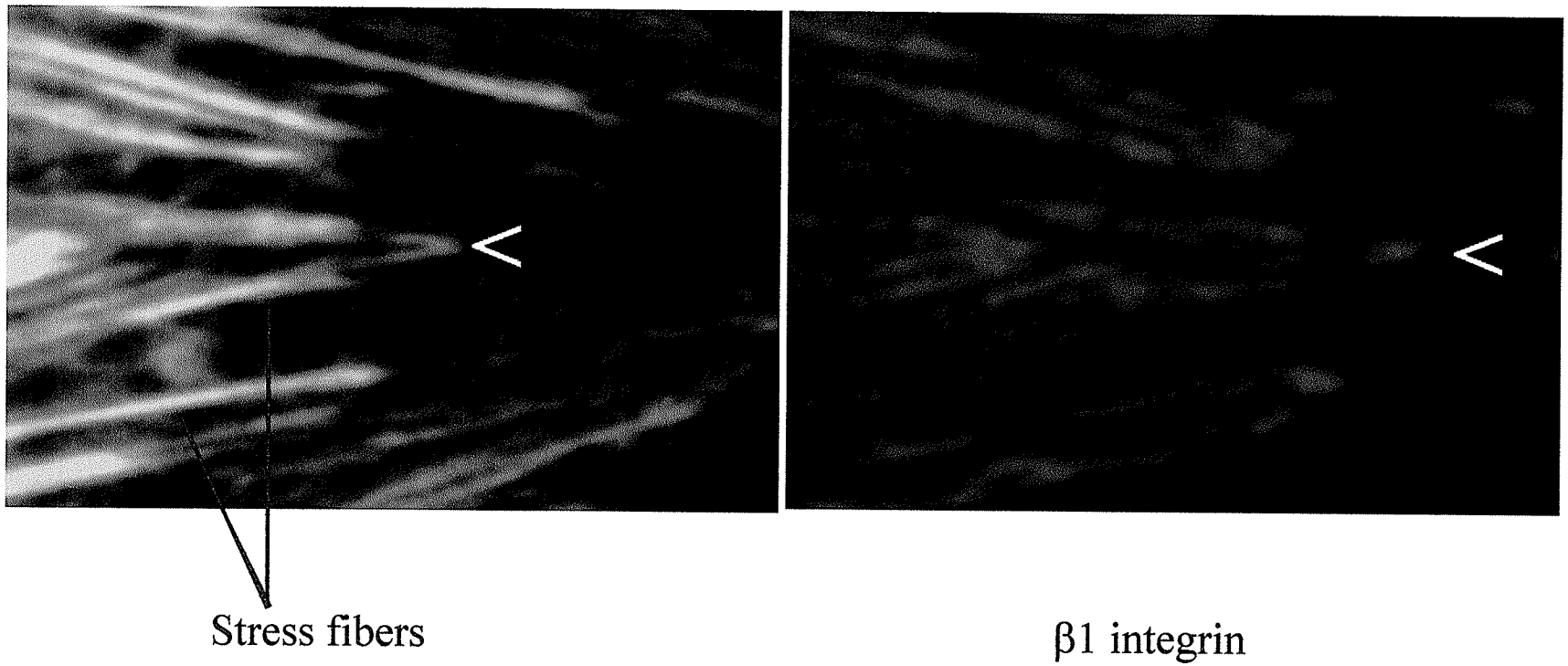

Fig. 1.8: Double staining of an adherent fibroblast for actin filaments (phalloidin-FITC) and β1 integrin (B3B11). Stress fibres originate from focal adhesion sites where integrins cluster (white arrows)

(ECM), stress fibers must be strongly anchored to the plasma membrane at the focal contacts. Attachment between actin filaments inside the cell and extracellular matrix on the outside of the cell is mediated by integrins. External domain of integrin binds to ECM ligands and its cytoplasmic domain linked to actin filaments in stress fibers by a multitude of proteins called focal adhesion proteins.

1.7. Focal Adhesion Complexes

Focal adhesions are formed in close apposition with the substratum where actin stress filament ends are anchored to the plasma membrane (Burridge et al, 1988). Focal adhesions can be readily visualized by reflection contrast microscopy in cells grown on ECM, appearing as spear tip-like structures connecting the ECM with the ventral plasma membrane (fig. 1.9; Jockos et al, 1995). Attachment of cells to extracellular matrix results in the co-localization and clustering of integrin receptors (fig. 1.10) in to supramolecular complexes with structural, cytoskeletal and signaling molecules (Chen et al, 1986; Burridge and Chrzanowska-Wodnicka, 1996; Damsky et al, 1985; Giancotti et al, 1986). Transfection experiments with chimeric proteins demonstrated β, but not α subunit cytoplasmic domains localize at focal adhesions (LaFlamme et al, 1992). This suggests β cytoplasmic sequences contain necessary information to recognize components of the focal adhesions. The extracellular events required for focal adhesion formation involve integrin clustering in the plane of plasma membrane as well as ligand binding. Most of the intracellular responses can be triggered by antibody-induced integrin clustering at the cell surface (Miyamoto et

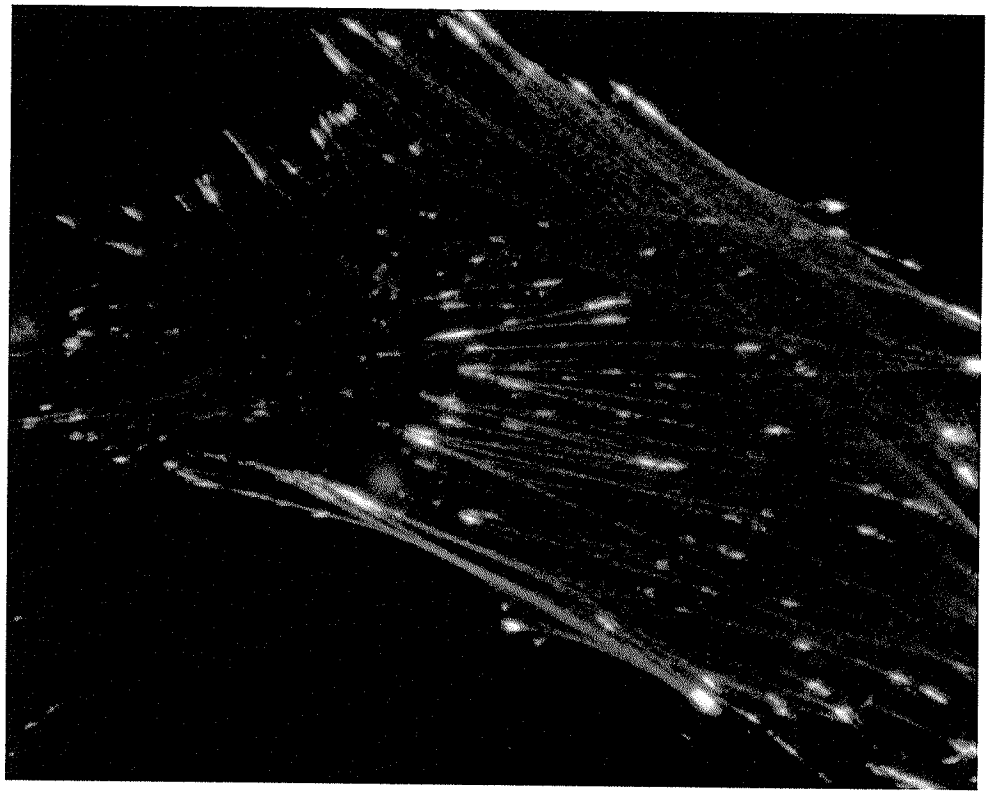

Fig 1.9: Focal adhesion complexes in an adherent fibroblast. Merged image of a double staining for F-actin decorated with phalloidin-FITC (green) and vinculin (red), a known focal adhesion protein

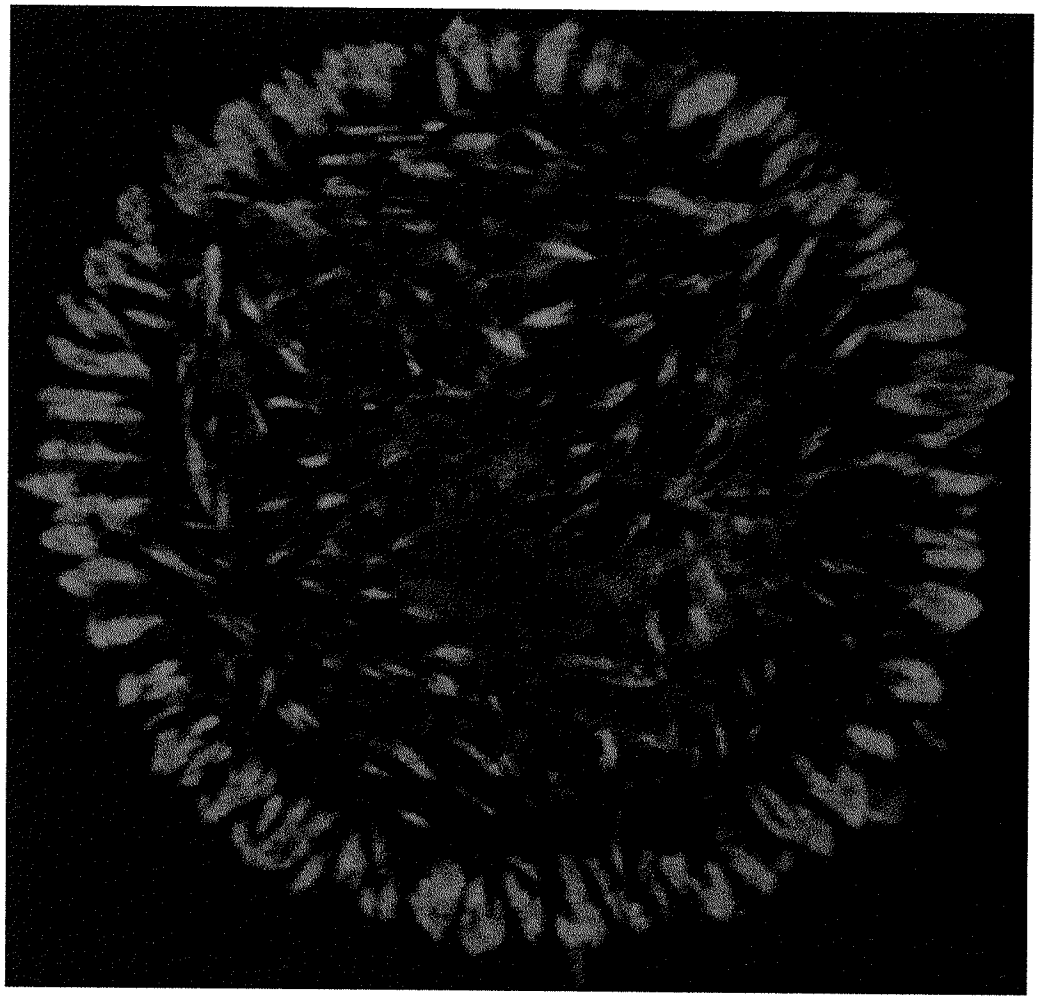

Fig. 1.10: Clustered integrin molecules at focal adhesion sites. Adherent cell grown on fibronectin coated slide was stained with anti-β1 integrin antibody.

al, 1995; Yamada and Miyamoto, 1995). When clustering was induced by antibodies, actin, talin, vinculin, α-actinin, Fak and tensin co-clustered with integrins. Thus clustering of integrins at the cytoplasmic face is an early and necessary event in focal adhesion formation.

Focal adhesions serve two cellular functions: to transmit tension at adhesion sites to maintain strong attachment to the underlying ECM and to act as signaling centers from which numerous intracellular pathways emanate to regulate cell's migration, growth, survival, apoptosis and gene expression (Sastry and Burridge, 2000; Schwartz et al, 1995; Howe et al, 1998; Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 1992; Jockos et al, 1995). Cell motility requires the formation of new focal adhesions at the leading edge of the cell and their breakdown at the rear of the cell (Petit and Thiery, 2000; Hynes, 1992). Loss of adhesion to ECM can lead to cell cycle arrest or apoptosis (Ruoslahti and Reed, 1994). Studies have shown focal adhesions are richly populated with catalytically active signaling proteins. For example, protein tyrosine kinases such as Fak, Src (and Src family members), Csk and Ab1; the serine/threonine kinases PKCα and δ and; the tyrosine phosphatases, LAR, PTP1B (Taylor et al, 1998; Burridge and Chrzanowska-Wodnicka, 1996; Jockos et al, 1995). The large repertoire of signaling proteins present in focal adhesions underscore the importance of these structures in propagating signals (Burridge and Chrzanowska-Wodnicka, 1996).

Our current knowledge is limited about the mechanisms or structural properties that recruit proteins to the focal adhesion complexes. All integrins except

β4 have very small cytoplasmic domains that allow accommodating a maximum of about two cytoplasmic proteins (Defilippi et al, 1997). Thus, the triggering of signaling pathways and recruitment of molecules require involvement of docking/adapter proteins. Proteins such as vinculin, Fak, paxillin and tensin may perform this function as they contain a number of docking sites or domains mediating protein-protein interactions. For example, vinculin can interact with at least 10 other focal proteins in the complex (Geiger and Bershadsky, 2001). Focal adhesion proteins such as α-actinin, talin and Fak appear to interact with the cytoplasmic tail of integrin (Otey et al, 1993; Knezevic et al, 1996 Schaller and Parsons, 1995). Talin, α-actinin, filamin, radixin, tensin, vinculin, gelsolin, profilin, VASP and Mena bind to actin (Burridge and Chrzanowska-Wodnicka, 1996; Jockos et al, 1995). Additional focal adhesion proteins including paxillin, zyxin, and cysteine rich protein (CRP) bind to neither actin nor integrin but may localize to the focal adhesion indirectly by binding to actin/integrin-binding proteins (Brown et al, 1996; Arber and Caroni, 1996; Beckerle, 1986 Sadler et al, 1992). Several focal adhesion proteins contain Src homology-2 (SH2) domains (Src, tensin, p85PI3K and Grb2) and SH3 domains (Src, PI3K, Grb2, P130 and Fak). Up on adhesion to ECM, a number of focal adhesion proteins become tyrosine phosphorylated and interact with SH2 containing proteins. Other proteins such as paxillin, vinculin and Fak contain proline rich sequences, which serve as docking motifs for SH3 containing proteins (Taylor, 1998).

Focal adhesions are dynamic structures that are formed on demand and disperse. For instance, they assemble, disassemble and recycle as cells migrate or

enter into mitosis (Sastry and Burridge, 2000), suggesting they are tightly regulated. Therefore, it is not surprising that many of the focal adhesion proteins exhibit low affinity binding with partners or exists in minute quantities. Because they are multiprotein heterogeneous complexes, their assembly requires that individual focal adhesion proteins interact with multiple binding partners (fig. 1.11). The assembly of focal adhesions is undoubtedly complex. How are proteins recruited to the complex, what are their temporal and spatial distributions? Moreover, how is assembly and disassembly of such structures regulated? Since these proteins (focal adhesion proteins) control most of what integrins do, answers to these questions will likely reveal how integrin mediated cellular adhesion processes are controlled.

1.8. Cell Migration

Cell migration plays a central role in normal physiology and pathology. Migration of fibroblasts and vascular endothelial cells is essential for wound healing and tissue repair. In metastasis, tumor cells migrate from the initial tumor mass into the circulation to reach new sites. Cell migration is a complex process requiring co-ordinated activity of cytoskeleton, membrane and adhesion molecules. This process can be viewed as having four distinct stages: protrusion, adhesion, traction and retraction (Fig. 1.12).

Forward movement of membrane at the leading edge of the cell is called protrusion. Extension of protrusive structures such as lamellipodia and filopodia are universally found in migrating cells. Lamellipodia are organelle free broad, flat sheet-

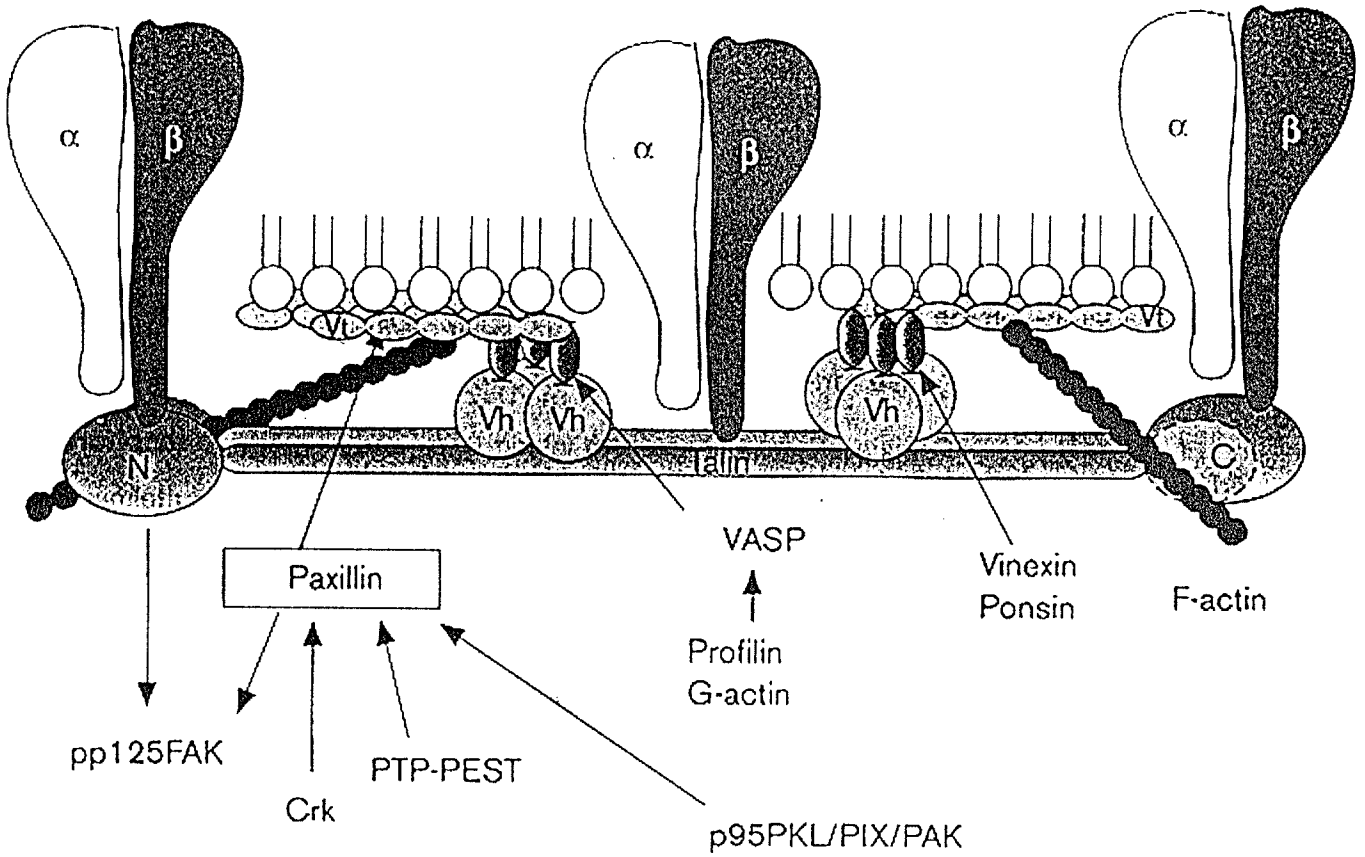

Fig. 1.11: Molecular interactions in focal adhesions (Critchley, 2000)

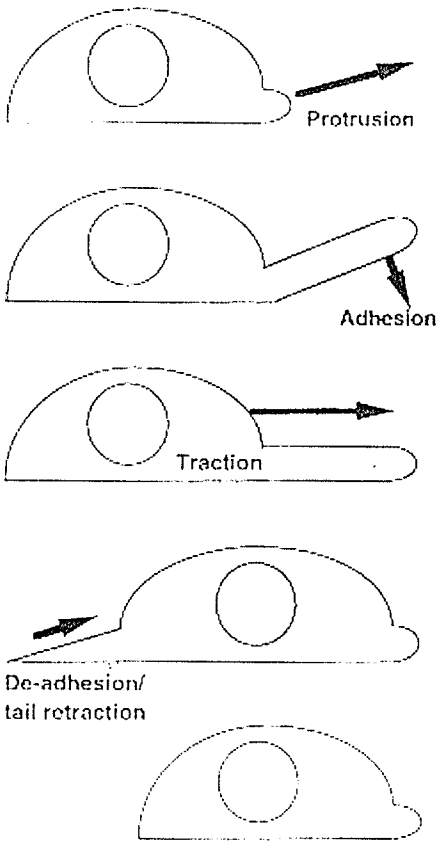

Fig. 1.12: Four stages of cell migration. (Mitchison and Cramer, 1996)

like structures while filopodia are thin, cylindrical, projections, both containing dense array of actin filaments. These are dynamic structures formed from a steady state of local actin polymerization at the leading edge of the migrating cells (Cortese et al, 1989) while depolymerizing at the rear (Wang, 1985). Lamellipodia may have smooth or serrated anterior edges appear to glide forward, pulling the cell body passively behind them (Bray, 1992).

As the protrusive structures move forward, they establish at the new location by adhesion on to the underlying surface (Hynes, 1992), permitting net forward progress. Integrin mediated new adhesions occur at the leading edge of protrusive structures (Regen and Horwitz, 1992). New focal adhesions form at the cell front and they increase in size and numbers as the cell migrates over them, persisting on the substratum until they reach the rear (Izzard and Lochner, 1980). Exocytosis (Hopkins et al, 1994) and surface directed movement (Schmidt et al, 1993) were implicated as the means of providing fresh supply of integrin molecules to the leading edge of the cell. Actin bundles are attached to the new focal adhesion contacts, which anchor the bundles to the plasma membrane. Focal contacts facilitate attachment of the cell to the substratum, and remain in place as the cell move over them confirming them as the points of traction (Lauffenburger and Horwitz, 1996). Increased concentrations of cytoskeletally-associated components are also enriched in the leading edge (Wu and Goldberg, 1993; Nobes and Hall, 1995). Upon adhesion, focal adhesion proteins such as Fak, paxillin and tensin are tyrosine phosphorylated (Lo et al, 1994; Schaller and Parsons, 1994; Turner, 1994).

As stated in the foregoing, the first force needed by a migrating cell is the protrusive force and is mediated by actin polymerization independent of myosin motor activity. Second force is the contractile force needed to move the cell forward that is dependent on myosin motor activity (Chen, 1981). Traction, the process of moving the cell body forward is mediated by the contraction of myosin II in stress fibers. Contractions at the junction between lamellipodia and cell body and at the rear of the cell deform the cell body and propel its internal contents forward toward the leading lamella. The major function of myosin II based contraction in migrating cells is to break the adhesive interactions. Myosin II mediated contraction pull on filaments connected to integrin receptors that are linked to ECM. This force disrupts the bonds at the extracellular integrin-ligand site and/or at an intracellular integrin-cytoskeleton site.

Cell migration also requires efficient mechanisms to release adhesions at the rear of the cell. Major fraction of integrin being left on the substratum as the cell releases and moves forward has been demonstrated (Regen and Horwitz, 1992). Cytoskeletally associated proteins such as talin and vinculin were not present in these remnants of membrane ripping. In the rear of the cell, the integrin-cytoskeletal linkage tends not to form and the membrane is less well supported by the cytoskeleton. In contrast, in the cell-front, the integrin-cytoskeletal linkage does form and the membrane is well supported. Schmidt et al (1993) have shown that at least four-fold difference in cytokeletal linkages between the front and rear of migrating cells.

Three Rho GTPases namely Cdc42, Rac and Rho were identified as important regulators of different adhesion structures formed in migrating cells. These are members of the Rho subfamily of Ras family GTP binding proteins. These molecules regulate the formation of filopodia, lamellipodia, focal adhesions and stress fibres (Hall, 1994). Formation of filopodia is regulated by Cdc42 (Kozma et al, 1995), while that of lamellipodia is regulated by Rac (Ridley et al, 1992). Focal adhesions containing stress fibre termini are regulated by Rho (Ridley and Hall, 1992). Rho has also been implicated in adhesive release at the rear end of the cell, inactivation of which has inhibited migration (Miura et al, 1993; Paterson et al, 1990).

1.9. Mass Spectrometer

A mass spectrometer is an instrument that produces ions and separates them in the gas phase for analysis (Siuzdak, 1994). Mass analysis is essentially separation of ions according to their mass to charge ratio, m/z, where m and z are mass and charge respectively. Tandem mass spectrometers use this separation as a preparative tool to isolate an ion with a specific m/z for further analysis (McLafferty, 1981). Further analysis is carried out by fragmenting the mass-selected ion and by determining the m/z of the fragment ions in a second stage of mass analysis.

A typical mass spectrometric analysis is made up of the following steps:

Sample Introduction → Ionization → Mass Separation → Ion Detection/Data Analysis

A fundamental challenge to the application of MS to any analyte is the production of gas-phase ions. Difficulties in producing gas phase ions can prevent

MS analysis of certain classes of molecules. The process of transfer of non-volatile molecules to charged molecules (ions) into the gas phase has been accomplished by many ways. They range from simple electron-impact ionisation and chemical ionisation to a variety of desorption ionisation techniques such as electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI). MALDI was introduced (Karas and Hillenkamp, 1988) as a soft ionisation technique for high molecular weight biomolecules such as proteins and peptides. For MALDI, the analyte is dissolved in a solution of UV-absorbing compound called matrix and placed on a stage in the mass spectrometer. As the solvent dries, matrix crystallises and the analyte molecules are included into the matrix crystals. Then a pulse of UV laser beam is used to desorb and ionize small amounts of the co-crystallised matrix and analyte from the surface of the stage (fig. 1.13). The matrix (e.g., 2, 5-dihydroxybenzoic acid) also serves to minimize sample damage from the laser beam by absorbing the incident laser energy, resulting in the sample and matrix molecules being ejected into the gas phase. The actual mechanism of MALDI, a combination of desorption and ionization, is still being investigated. The laser energy absorbed by the matrix leads to intense heating and generation of a plume of ejected material. Generation of ions is believed to arise through ion/molecule reactions in the gas phase. Nevertheless, enough energy is transferred to the molecule to generate protonated peptide ions, $[M+H]^+$ in the positive ion mode. Acidic environment provided by the acidity of most matrix compounds facilitates this process. The sample ions that are formed during this process are either singly or multiply charged. Singly

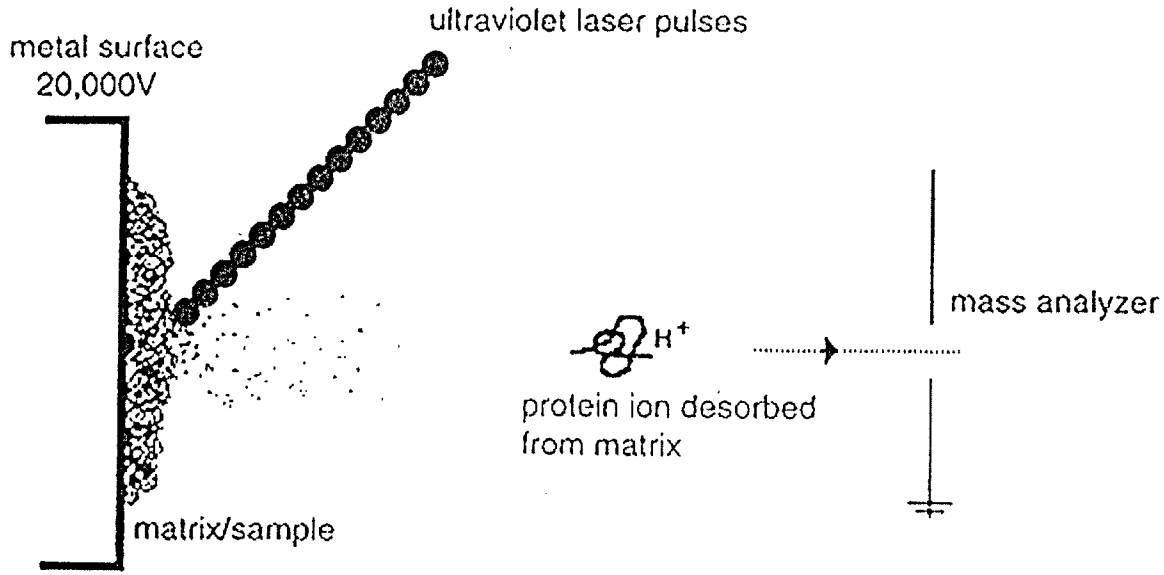

Fig. 1.13: MALDI source (Suizdak, 1994).

charged ions are dominant in MALDI mass spectrum. Once ions are formed in the gas phase, they are electrostatically directed to the mass analyzer, which differentiates the ions according to their mass-to-charge ratio (m/z).

As stated above, mass spectrometers determine the m/z of the ions derived from the analyte. The molecular weight of the analyte can be calculated from its m/z, provided the z is known. A variety of mass analysers are available to make this measurement. Time of Flight (TOF) is one such analyser. TOF mass spectrometry was first successfully used as an ion analyser in the 1950's (Wiley and McLaren, 1955). The principle of mass analysis by TOF analyser is simple. An ion is given a fixed amount of kinetic energy by acceleration in an electric field that is generated by the application of a voltage. This acceleration causes the ion to travel at a velocity that is inversely proportional to its m/z. Thus, the time required for each ion to travel the flight tube is mass dependent. High mass ions take longer to reach the detector than low mass ions. Several ionisation techniques are suitable for TOF mass analysers, provided the ions are generated or ejected from the ion source over very short periods of time. One of such ionisation methods that is well suited for TOF is MALDI. Linear TOF MS instruments are capable of attaining a resolution of 1 part per 1000. Its most important limitation is the energy spread of the ions. However, in reflection TOF instruments (fig. 1.14), electrostatic mirrors provide energy focusing and are thus capable of a resolving power of over 10,000. High mass range and efficiency are two advantages of TOF analysers. Parallel detection and lack of slits

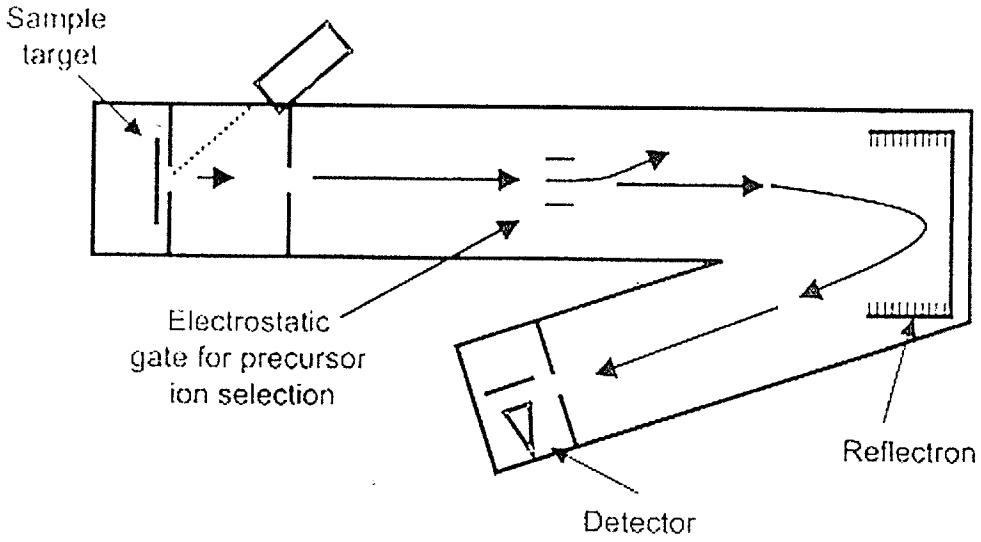

Fig. 1.14: Reflection Time of Light instrument (Kinter and Sherman, 2000).

cause rapid ions accelerated out of the ion source of the TOF instrument to reach the detector with high efficiency.

Since most MALDI instruments are used in conjunction with the time-of-flight analysers (TOF), mass range is practically unlimited, except for problems of ion production and detection. MALDI is perhaps the most forgiving of all the ionisation techniques with respect to the presence of salts and other contaminants in the sample in terms of interfering with the ion production. Peptide mass-mapping experiments are the most common application of MALDI-TOF to proteomic research. It is extremely sensitive in being able to detect few fmols of peptide. One potential problem of MALDI MS during the analysis of small peptides (< m/z 600) is the high background generated from the matrix. Another disadvantage is that the analyte-matrix adduct ions are formed which can complicate the spectrum. Ion suppression effect also exists with this technique.

A mass filter is required to select a single parent ion for tandem mass spectrometry. Quadrupole mass filter is a widely used type because of its ease of use, small size and relatively low cost (Chernushevich et al, 2001; Andersen and Mann, 2000). Paul and Steinwedel (1953) published the basic principles of the quadrupole mass filter. Mass separation in a quadrupole mass filter is based on achieving a stable trajectory for ions of specific m/z values in a hyperbolic electrostatic field. A quadrupole mass filter consists of four parallel cylindrical rods (fig. 1.15). Opposite rods are connected in pairs to both radio frequency (RF) and direct current (DC) generators. Thus, the ions during their passage through the rods will be bathed in the

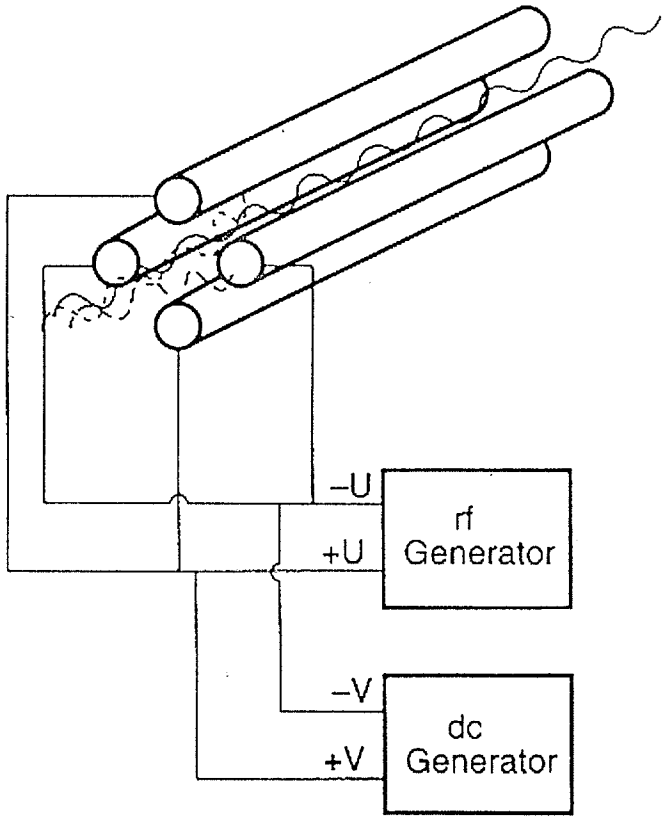

Fig. 1.15: Quadrupole mass filter. (McCloskey, 1990)

combined electric and radio frequency fields. The output of the RF generator is energy in the radio frequency part of the electromagnetic spectrum, and is a sinusoidal voltage signal with amplitude U and frequency ω. The output of the DC generator is two different voltages +V and –V.

At given values of U and V, only certain ions will have stable trajectories and are allowed to pass through the rods to the detector. All other ions will have unstable trajectories and will be lost. In other words, the range of ions of different m/z values, capable of passing through the mass filter, depends on the ratio of U to V. One of the advantages of a quadrupole mass filter is the low voltage applied to the ion source. This eliminates high voltage problems and makes interfacing to GC and LC easier.

1.10. Protein Identification by Mass Spectrometry

Recent advances in instrumentation and techniques of ionisation have enormously popularized the use of mass spectrometry in biological sciences (Peng and Gygi, 2001; Chace, 2001). These advances permit the accurate determination of the molecular masses of large macromolecules. Today (Hakansson et al, 2001; Yeung et al, 2001), substances in the attomole/μl range can be measured often using small and impure biological samples. Mass spectrometry thus has become an indispensable tool for peptide and protein analysis (Mann et al, 2001).

The general approach of mass spectrometry based protein identification has been as given below.

1) Generation of peptides by digesting protein with trypsin:

Protein sequencing and identification is best accomplished by sequencing peptides derived from a protein rather than the intact protein. Therefore, the first step is to cut the protein into a series of peptides using trypsin. Trypsin, being a restriction endopeptidase cuts the amide bond at the C-terminal side of Lysine (K) and arginine (R) residues, except when they are placed before proline. K and R are common residues (5-6%) in mammalian proteins and trypsin digestion results in the generation of 1000-2000 Da peptides, which are ideal for analysis in MALDI-TOF machines. The second reason for using trypsin is to place basic residues at the C-terminus of the peptides. The presence of basic residues at the C-terminus results in a more predictable fragmentation throughout the length of the peptide. Arginine if present in the middle of a peptide will often result in the absence of fragmentation at several contiguous peptide bonds adjacent to the arginine (http://abrf.org/ABRF/Research-Committees/masspecquiz/SequencingTutorial.html).

2) Acquisition of a single MS spectrum and subsequent identification of the protein using peptide mass data (peptide fingerprint):

Protein identification is done with a database search program. The program performs *in silico* digestion of the proteins in the database to create their peptide fingerprint and compares them with the input peptide fingerprint to identify the protein (Blackstock, 2000).

3) Tandem mass spectrometry:

A single ion mass (parent ion) is selected from the MS spectrum using a mass filter. The selected ion is then transmitted to a high-pressure region of the instrument where it undergoes collision-induced dissociation (CID) with gas molecules. This results in the cleavage of peptide backbone and the formation of a series of ions called product ions (daughter ions). These ions contain the partial sequences of the peptide from which the aminoacid sequence of the peptide, or at least a large part of it, can usually be deduced (fig. 1.16). However, the tandem MS data is routinely interpreted using database search programs. The program utilizes the product ion data to search the database of theoretical spectra derived from the proteins in the database. Thus it deduces the aminoacid sequence of the peptide and hence the identity of the protein from which the peptide was derived.

The use of mass spectrometry in biological sciences has grown enormously, with applications to all areas concerned with events occurring at the molecular level. MALDI-MS has emerged as an effective bioanalytical tool (Biemann, 1992; Chait et al, 1992) and the fact that it allows the analysis of heterogeneous samples makes it attractive for biological samples (Bahr et al, 1997). MALDI technique has been extremely useful in the analysis of peptides. One of its common applications is the identification of protein by analysing peptide mixtures produced by protease digestion. Similar to the current project, others have also used MS based protein identification on large protein complexes (Neubauer et al, 1997; Mann et al, 2001). The reproducibility of such an approach (purification of large complexes) has been reported as 70% (Gavin et al, 2002). The 30% detected loss of association was

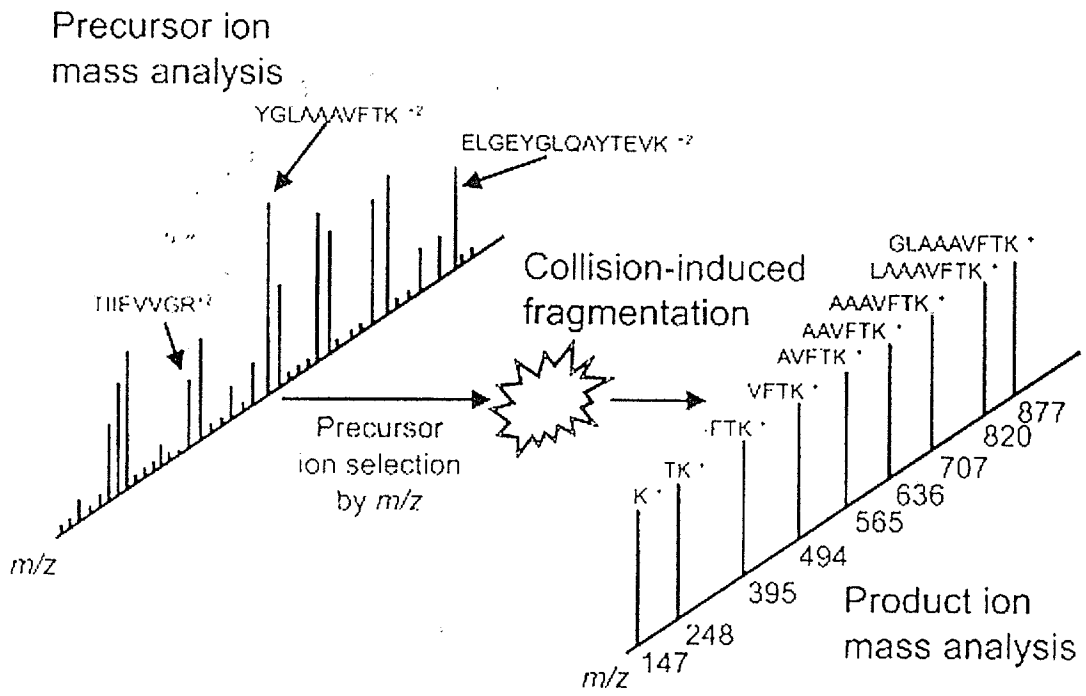

Fig. 1.16: Sequencing of mass selected peptide ion by tandem mass spectrometry (Kinter and Sherman, 2000).

attributed to the inherent variability of the technique such as biological samples, purification and mass spectrometry. This calls for 30% of any association observed by this study to be treated with caution. In other words, any new/previously undocumented molecule detected in the adhesion complex by the present study to be repeatedly demonstrated by conventional techniques. Nevertheless, MS based protein identification offers us an opportunity to identify a protein molecule without requiring prior knowledge about it or specific reagents to it (e.g., antibody). Unlike ESI-MS, for instance, MALDI-MS does not require pure protein samples. These features were ideal for the current project to identify known/unknown protein molecules in adhesion complexes.

2. MATERIALS AND METHODS

2.1. Sources of Materials

Anti-G3BP, mouse monoclonal antibody (clone 23): Transduction Laboratories,

Anti-RasGAP, mouse monoclonal antibody (B4F8): Santa Cruz Biotechnology,

Anti-RasGAP, rabbit polyclonal antibody (Sc-425): Santa Cruz Biotechnology,

Anti-human CD3, mouse monoclonal antibody (UCHT1): Pharmingen,

Anti-human β1 integrin, mouse monoclonal antibodies,

3S3 (Gao and Wilkins, 1995)
N29 (Wilkins et al, 1996)
B3B11 (Wilkins et al, 1995)

Cyanogen bromide (CNBr) activated Sepharose 4B beads: Pharmacia,

Tosyl activated magnetic beads (diameter 4.5μ): Dynal,

Latex beads (diameter 6.2μ): Sigma,

Complete protease inhibitor cocktail: Boehringer Mannheim,

Slowfade mountant: Molecular Probes,

Excision grade trypsin: Calbiochem

2.2. Cell Lines

K562 and Human foreskin fibroblast (HFF) cell lines were cultured and maintained in RPMI 1640 with 10%FCS in a 5% CO_2 atmosphere at 37°C. HFF cell line: Kind gift from Doug Milley, Cadham Provincial Laboratory, Winnipeg, MB

2.3. Isolation of Integrin Associated Molecules by Fishing Cell Lysate

2.3.1. Antibody Coupling of CNBr Activated Sepharose Beads

1. Dialyze N29 antibody against 0.1M sodium bi-carbonate buffer containing 0.5M NaCl, pH8.3 (coupling buffer)
2. Swell and wash the sepharose beads in 1mM HCl. Perform a quick final wash in coupling buffer
3. Mix 1ml (packed volume) of beads with 1.2 mg of N29 antibody and incubate at room temperature for 4 hours in an end-over-end mixer
4. Wash the beads twice in coupling buffer
5. Block the beads in 1M ethanolamine pH8.0 for overnight at 4°C
6. Strip the beads by washing alternatively with 0.1M acetate buffer containing 0.5M NaCl, pH4.0 and 0.1M Tris-HCl containing 0.5M NaCl, pH8.0 (3 cycles)
7. Store the beads in TBS containing 0.02% sodium azide at 4°C

2.3.2. Preparation of K562 Cell Lysate

1. Wash $3X10^8$ K562 cells three times in cold PBS
2. Add 3ml of lysis buffer (50mM HEPES-KOH pH7.3, 10% glycerol, 1mM sodium fluoride, 1mM DTT, complete protease inhibitor cocktail, 60mM potassium acetate, 0.5% BSA, Zachariae et al, 1998)
3. Grind cells 30 minutes using a 7 ml Wheaton's homogenizer in an ice bath

4. Sonicate twice for 15 seconds with an output control setting of 3.5 (Vibra Cell sonicator, Sonics & Materials Inc. Danbury, Connecticut).

5. Centrifuge at 15,000g for 15 minutes at 4°C and collect the clear supernatant

2.3.3. Preparation of Human β1 Integrin

1. Homogenize human placenta in TBS containing 2mM $CaCl_2$, 50mM n-octylglucopyranoside and 1mM PMSF (homogenization buffer)

2. Remove the solid particles and fat by centrifugation followed by passage through a cheese cloth

3. Mix the homogenate with 3S3 antibody coupled sepharose beads and incubate at 4°C for overnight in a rocker

4. Pack the beads in to a column and wash with at least 20 column volumes of homogenization buffer

5. Elute β1 integrin using 100mM glycine-HCl, pH2.5 containing 1mM $MgCl_2$, 0.1mM $CaCl_2$ and 50mM n-octylglucopyranoside. Neutralize the elutes immediately with 2M Tris-HCl, pH8.2

2.3.4. β1 Integrin Ligand Binding Assay

1. Dilute human β1 integrin (1μg/ml) in integrin buffer (25mM TBS containing 0.1% $CaCl_2$ and 1mM $MgCl_2$). Pipette 100μl/well of the diluted integrin in to a 96-well plate (*Nunc maxisorb*) and incubate at 4°C overnight

2. Wash the wells 3 times in wash buffer (PBS with 0.05% Tween20)

3. Block the wells with block buffer (200µl/well) for 1 hour at room temperature (block buffer: 10% fibronectin denatured FBS in integrin buffer)

4. Perform a serial doubling dilution of the biotinylated-fibronectin from 25µg/ml to 0.844µg/ml in dilution buffer (1/10 block buffer). Pipette 100µl/well of the diluted biotinylated-fibronectin and Incubate at 30°C for 3 hours. Wash 3 times with wash buffer

5. Pipette 100µl/well of 1/4000 avidin-alkaline phosphatase (Sigma) in dilution buffer

6. Incubate for 30 minutes at 37°C and wash 3 times with wash buffer

7. Pipette 100µl of substrate (0.1% p-nitrophenylphosphate in 0.2M Tris buffer, Sigma) and incubate at 37°C for color development

8. Measure the OD at 405nm Vs 690nm

2.3.5. Isolation of Integrin Associated molecules

1. Label three 1.5 ml Eppendorf tubes as "Test", "Integrin control" and "Lysate control"

2. Pipette 40µl of N29-coupled beads in each of the 3 tubes

3. Add 60µl (60µg) of human β1 integrin in "Test & Integrin control" tubes. To "Lysate control" add 60µl of integrin-buffer

4. Incubate in a rotator at 4°C for overnight

5. Wash the beads twice in integrin-buffer
6. Pipette 1ml of fresh K562 cell lysate each to "Test & Lysate control" tubes and 1ml lysis buffer to "integrin control" tube
7. Incubate at room temperature for 30 minutes in a end-over-end mixer
8. Wash 3 times in cold lysis buffer
9. Elute the beads in 100µl of elution buffer (100mM glycine-HCl pH 2.5) at room temperature for 30 minutes
10. Run on a 10% SDS-poly acrylamide gel under reducing conditions
11. Silver stain the gel

2.3.6. Silver Staining (Shevchenkoet et al., 1996)

Fix the gel for 30 minutes with gentle shaking in fixation solution (45% methanol and 5% acetic acid in water)

Rinse the gel in deionised water for overnight

Sensitize in 0.02% sodium thiosulfate for 1-2 minutes

Rinse in two changes of demonized water, 1 minute each

Incubate the gel in chilled 0.1% silver nitrate solution for 30 minutes at 4°C with shaking

Rinse the gel in two changes of deionized water, 1 minute each

Develop in developer (0.04% formalin and 2% Na_2CO_3 in deionized water)

Stop reaction by washing with 5% acetic acid

2.4. Isolation of the Adhesion Complex Proteins from VPM Preparation

2.4.1. Preparation of Ventral Plasma Membrane by Wet Cleaving Method

(Brands and Feltkamp, 1988)

1. Immerse coverslips (sulfuric acid washed and sterile) in fibronectin solution (10μg/ml in PBS) and leave at 4°C for overnight (fibronectin coating)
2. Rinse the fibronectin-coated coverslips in sterile PBS
3. Layer 300μl of cell-suspension on each of the coverslips
4. Incubate at 37°C in a CO_2 incubator for overnight
5. Rinse the coverslips in VPM buffer (25mM Tris, 135mM NaCl, 5mM KCl, 0.5mM NaH_2PO_4, 1mM $CaCl_2$, 0.5mM $MgCl_2$, pH7.2)
6. Overlay the cell-sheet with a piece of nitrocellulose membrane (0.45μm) that is pre-wet in VPM buffer and semi-air dried. Leave it for 5 minutes
7. Cleave the cells by gently lifting the membrane using a pair of forceps
8. Rinse the VPM in integrin-buffer

Note: For biochemical analysis, VPM preparation was made in fibronectin-coated petridishes instead of fibronectin-coated coverslips. The material was then dissolved in hot gel loading buffer (reducing) to be later resolved by SDS-PAGE.

2.4.2. Immunofluorescent Staining of VPM (Brands and Feltkamp, 1988)

1. Rinse the VPM in wash buffer (10mM sodium phosphate, 100mM KCl, 2mM $MgCl_2$, pH7.0)

2. Fix the VPM in 4% PFA in PBS and rinse well in wash buffer
3. Incubate with the primary antibody diluted in wash buffer at room temperature for 1 hour, rinse in wash buffer
4. Incubate with secondary antibody diluted in wash buffer at 4°C for 1 hour and rinse in wash buffer
5. Mount the coverslip, face down on a microscope slide using *Slowfade* mountant

2.5. Isolation of Adhesion Complexes Using Antibody Coupled Magnetic Beads

2.5.1. Antibody Coupling to Tosyl Activated Magnetic Beads

1. Wash beads twice in 0.1M phosphate buffer pH7.4 (coupling buffer)
2. Incubate 100 µg of antibody with $3X10^8$ of beads (in 750µl total volume of coupling buffer) at 37°C for 24 hours with slow rotation.
3. Wash the beads twice in PBS containing 0.1% BSA.
4. Block free tosyl groups in 0.2M Tris buffer pH8.5 containing 0.1% BSA with slow rotation at room temperature for 24 hours
5. Wash the beads once in PBS containing 0.1% BSA and store in the same buffer containing 0.02% sodium azide at 4°C

2.5.2. Antibody/Poly-L-Lysine Coating on Latex beads

Magnetic beads showed auto-fluorescence in both red and green channels. Therefore, for immunofluorescent microscopy work, antibody/poly-L-lysine (PLL) was coated on to latex beads instead of magnetic beads

1. Mix 1 ml of 1% latex bead-suspension with 2 mg of antibody/PLL in 25mM MES buffer, pH6.1
2. Incubate in a rotator at 4°C overnight.
3. Block in 1% BSA in MES buffer at room temperature for overnight
4. Store the beads in MES buffer containing 0.02% sodium azide at 4°C

2.5.3. Induction & Isolation of Adhesion Complexes (Plopper and Ingber, 1993)

1. Incubate $15X10^6$ K562 cells with $15X10^7$ 3S3-coated magnetic beads at 37°C for 30 minutes with slow rotation.
2. Isolate the bead-bound cells using a Dynal magnet block
3. Suspended the cell-bead pellet in ice-cold CSK buffer without detergent. (All the subsequent procedures were carried out in an ice bath).
4. Transfer the pellet in to 2ml of complete CSK buffer (0.5% Triton-X-100, 50mM NaCl, 300mM sucrose, 3mM MgCl2, 10mM sodium fluoride, 1mM sodium vanidate, complete protease inhibitor cocktail and 10mM PIPES pH6.8). CSK buffer first described by Burr et al, 1980 is able to preserve the integrity of cytoskeleton.

5. Sonicate the pellet for 10 seconds at a relative output setting of 0.4 (Ultrasonic 2000) and homogenize in a 7ml Wheaton's homogenizer (20 strokes). The beads were pelleted and washed five times in complete CSK buffer.
6. Boil the beads in gel loading buffer under reducing conditions and resolve the molecules on SDS-PAGE.
7. Silver stain the gel to visualize the protein bands.

2.5.4. In-Gel Digestion of Proteins for Mass Spectrometry

This method is as per Shevchenko et al., 1996 but with a modification. Formic acid was omitted during peptide extraction to prevent formylation of residues leading to misidentification (Rout et al., 2000).

1. Excise Individual protein bands from the silver stained gel and place them in labeled tubes containing 100mM ammonium bicarbonate solution
2. Wash bands in 50µl of the ammonium bicarbonate solution by brief vortexing
3. Spin the tubes at 500g for 1 minute, remove and discard the liquid
4. Wash the gel fragments in 3-4 gel volumes of 50% acetonitrile in 100mM ammonium bicarbonate for 15 minutes at room temperature. Spin briefly and discard the liquid.
5. Cover the gel fragments with acetonitrile. After the gel fragments have shrunk, spin briefly and remove acetonitrile.

6. Rehydrate the gel in 100mM ammonium bicarbonate. After 5 minutes add an equal volume of acetonitrile, wait for 15 minutes, spin briefly, remove and discard the liquid.

7. Dry the gel in a vacuum centrifuge for 5 minutes without heat

8. Reduce the cysteines residues in the protein by immersing the gel pieces in 2-3 gel volumes of 100mM ammonium bicarbonate containing 10mM DTT. Incubate for 1 hr at 56°C

9. Immerse the tubes quickly in ice-bath to bring the tubes to room temperature. Spin briefly and discard the liquid

10. Quickly add 2-3 gel volumes of 100mM ammonium bicarbonate containing 55mM iodoacetamide to alkylate the cysteines. Incubate in the dark for 45 minutes at room temperature with occasional vortexing. Spin and discard the liquid

11. Wash the gel fragments by vortexing in 100µl of 100mM ammonium bicarbonate

12. Shrink and dry the gel fragments as in step 4-7.

13. Digest the protein molecules in the gel by incubating with 2-3 gel volumes of digestion buffer on ice for 45 minutes (Digestion buffer: Excision grade trypsin, 5ηg/µl in 50mM ammonium bicarbonate containing 5mM $CaCl_2$). Spin briefly, and remove as much as liquid possible.

14. Fill the tubes with 5-20 µl of digestion buffer without trypsin and crush the gel pieces with a plastic applicator. Incubate overnight at 37°C

15. Spin briefly and collect the liquid to another labeled tube. Extract peptides by soaking the gel in 2-3 gel volumes of 25mM ammonium bicarbonate. Stand for 15 minutes with occasional vortexing, add an equal volume of acetonitrile and incubate for another 15 minutes. Spin briefly, collect the liquid and combine with the previous extract. Repeat extraction once more.

16. Add 10mM DTT solution to the combined extract to get a final concentration of 1mM.

17. Dry the combined extracts using vacuum centrifuge without heat

2.5.5. Removing Salts and Detergent from the Samples

1. Reconstitute the dried sample in 10µl of 0.1% TFA

2. Pre-wet the *ziptip* (Millipore) by aspirating wetting buffer (50% acetonitrile in water)

3. Equilibrate *ziptip* by washing 2 times in equilibration buffer (0.1% TFA in water)

4. Bind peptides to *ziptip* by aspirating and dispensing the sample 5-10 cycles

5. Wash *ziptip* by aspirating and dispensing in 0.1% TFA twice

6. Elute in 2-4 µl of elution buffer (50% acetonitrile in water) at least 3 cycles of aspiration and dispensing

7. Add equal amount of 0.2% TFA. The samples are now ready for MS analysis

2.5.6. MS Analysis of the Samples

All mass spectrometry analysis for this thesis was carried out using a tandem quadrupole/time-of-flight instrument with a matrix-assisted laser desorption ionisation source (MALDI-QqTOF) (fig. 2.1; Loboda et al., 2000, Shevchenko et al, 2000). For analysis of peptides in the molecular weight range up to at least 5000 daltons, it has a mass resolving power of about 10,000, accuracy of 10 ppm and sensitivity in the femtomole or less range (Loboda et al, 2000). This combination of TOF mass analyser with MALDI is an effective tool in the laboratory of biologists, due to their high sensitivity, speed and ease of operation. MALDI-QqTOF is a tandem mass spectrometer commonly employed to sequence peptides. Using this instrument a peptide mass map of the protein digest and tandem mass spectra of multiple peptide precursor ions can be acquired from the same sample in the course of one experiment.

MALDI-QqTOF tandem mass spectrometry uses two stages of mass analysis, one to preselect an ion for fragmentation and the second to analyse the fragments induced. This dual analysis is tandem in space and is accomplished with a combination of two mass spectrometers in series. In the QqTOF combination, Q represents a quadrupole mass filter; q, a rf -only quadrupole (collision chamber). An ion of interest generated in the ion source is preselected with the mass filter Q. This ion is then fragmented to produce daughter ions by collisions with an inert gas like argon or helium in the high-pressure collision chamber q. The fragmentation products are then analysed with the TOF mass analyser. This fragmentation process is termed

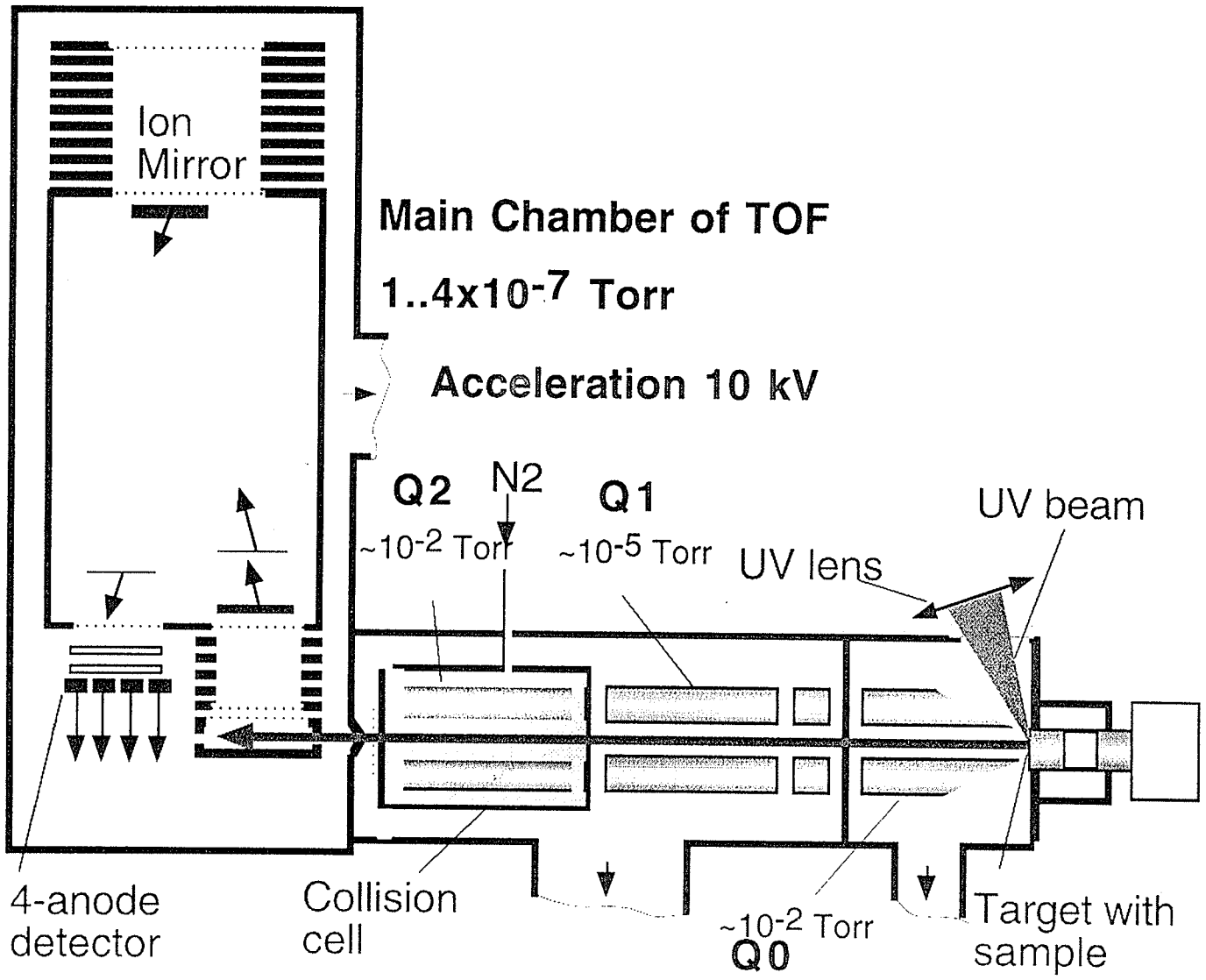

Fig. 2.1: MALDI-QqTOF. Courtesy: Time of Flight Lab, Department of Physics, University of Manitoba.

collision-induced dissociation (CID). In this way, it is possible to obtain sequence information from a peptide by analysing the backbone fragments with TOF analyser. The samples were analyzed as follows.

1. Place 0.6μl of dihydroxybenzoic acid (matrix) on one square area of the MALDI-chip and leave at room temperature to crystallize. The MALDI-chip has 40 squares and therefore up to 40 samples can be analyzed sequentially.
2. Add 0.6μl of sample on to the crystallized matrix and wait until dry. Load the chip on to the mass spectrometer and acquire data

Note: N_2-laser operating at 20Hz was focused on to the sample (spot size of 0.3 mm^2) to produce ions. To detect the singly charged ions, acceleration voltage of the TOF was raised to 4kV-10kV.

2.5.7. Data Acquisition and Analysis

1. Acquire the mass spectrum in single-MS mode
2. Analyze the single-MS data (peptide fingerprint) using *MS-Fit* at prospector.ucsf.edu with ±30 ppm peptide mass tolerance to identify the protein.
3. Select few peptides from the protein and subject them to tandem mass spectrometry. Analyze the tandem MS data using *MS-Tag* at prospector.ucsf.edu with ±30 ppm parent ion mass tolerance and ±50 ppm fragment ion mass tolerance.

Note: NCBInr protein database was used for both *MS-Fit* and *MS-Tag* of the above searches. A definitive identification is reached when at least three peptides confirm (by MS/MS) the identity of protein obtained by peptide fingerprint (MS) search (Shevchenko et al, 1996).

2.5.8. **Immunofluorescent microscopy** (LaFlame et al, 1992)

1. Mix $0.25X10^6$ HFF cells with 10^6 3S3-latex beads or PLL-latex beads and incubate at 37°C with slow rotary motion for 30 minutes.
2. Place few drops on a PLL coated (10μg/ml) coverslip and incubate for 30 minutes at 37°C in a CO2 incubator.
3. Rinse the cells in PBS and fix with 4% paraformaldehyde solution in PBS
4. Permiabilize the cells in 0.05% Triton-X-100 in PBS and rinse in PBS
5. Block with 1%BSA in PBS for 30 minutes at room temperature and rinse in PBS
6. Incubate the coverslip with primary antibody (biotinylated anti-G3BP or B3B11) diluted in PBS containing 0.1% BSA for 1 hour at room temperature and rinse in PBS
7. Incubate with avidin-FITC diluted in PBS containing 0.1% BSA at 4C for 30 minutes. Rinse and mount the coverslip face down on a microscope slide using *slowfade* mountant.

2.5.9. **Cell Wounding Experiment** (Kulkarni et al, 2000).

1. Grow HFF cells on fibronectin coated coverslips in 10% FBS containing medium until the cell layer becomes 70-80% confluent
2. Replace with serum free medium (serum starving) and incubate for another 24 hours for the cells to become 100% confluent
3. Make wounds by dragging a plastic yellow pipette tip across the cell layer, rinse and replace with serum free medium and incubate for 2 hours
4. Rinse the cell layer in PBS, fix in 4% PFA (15 minutes) and permiabilize in 0.2% Triton-X-100 in PBS (5 minutes) before staining with primary and secondary antibodies.

3. RESULTS

3.1. β1 Integrin Ligand Binding Assay

To test whether the β1-integrin preparation used in the following experiment was functional, a ligand-binding assay was performed using biotinylated fibronectin as stated in materials and methods. The assay showed that the integrin preparation was able to bind to its ligand (fibronectin) and that the binding was concentration dependent (fig. 3.1) indicating that the preparation is functional. It also proved that the protocol used for purifying β1-integrin from human placenta homogenate did preserve the functionality of integrin. Therefore, β1-integrin samples prepared likewise were used for fishing out integrin-associated molecules as given below.

3.2. A Fishing Strategy to Isolate Integrin Associated Molecules from Cell Lysate

As a preliminary attempt to isolate integrin-associated molecules, the cytoplasmic domain of β1 integrin was used to fish out molecules from fresh K562 cell lysate. This was done as described in materials and methods. Briefly, human β1 integrin was captured on to N29 coupled Sepharose beads. N29 antibody binds to residues 14-54 of β1 integrin (Wilkins et al., 1995) and therefore was the preferred antibody to capture and to orient integrin with free cytoplasmic tails available for binding molecules while fishing the lysate. This was then incubated with fresh K562 cell lysate to let molecules associate with β1 integrin's cytoplasmic tail. K562 is an erythrolymphoid continuos cell line that offers a good supply of cells in a short period

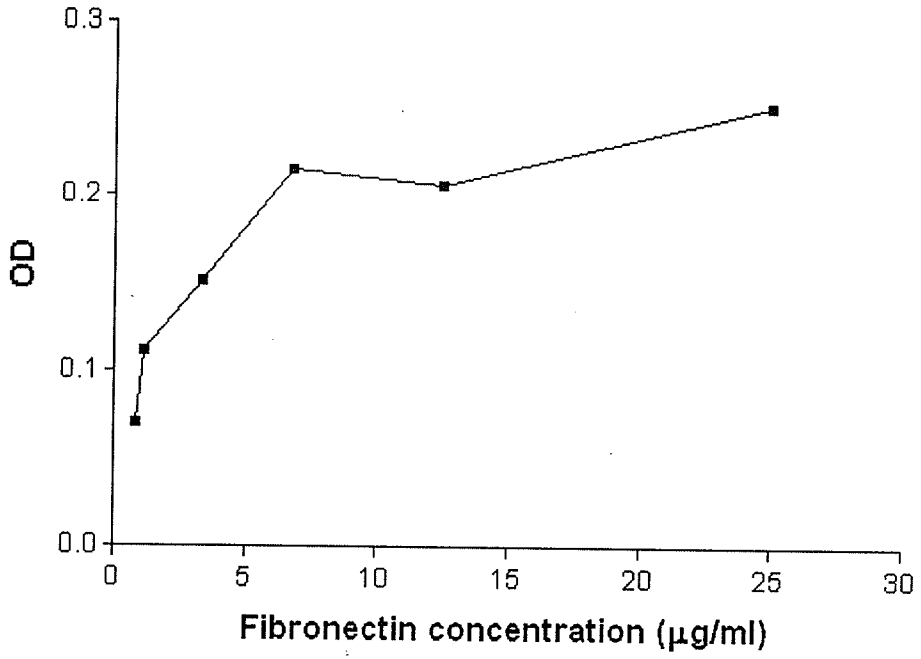

Fig. 3.1: Determining the functionality of β1 integrin by ligand binding. Six doubling dilutions of biotinylated fibronectin were incubated in ELISA plate coated with β1 integrin. The extent of ligand binding was then measured using a colorimetric assay

of time. Unlike primary cell cultures, it is homogenous with minimal inconsistency within the line. The isolated material was boiled to dissolve in gel-loading buffer and the molecules resolved by SDS-PAGE under reducing conditions. Silver staining was performed to visualize the isolated molecules and it revealed no new or intense bands in the test lane in comparison to the control lanes (fig 3.2). This approach was not pursued further because of its failure to provide any meaningful protein bands on silver stained gel to be analyzed.

3.3. Isolation of Focal adhesion Complexes Using Ventral Plasma Membrane

The second approach was to prepare ventral plasma membrane (VPM) from adherent cells and to isolate focal adhesion protein molecules for identification by mass spectrometry. HFF cells (a semi-continuos cell line) were used for this experiment because of their tight adhesion property. Focal adhesion structures form at the ventral side of an adherent cell where it makes contact with the extracellular matrix. Clustered integrins and focal adhesion proteins constitute these supramolecular complexes.

To optimize conditions of VPM preparation and to check the integrity of adhesion complexes in VPM, cells were grown on fibronectin-coated coverslips and VPMs were made by wet cleaving (Brands and Feltkamp, 1988). Briefly, a piece of semi-dry nitrocellulose membrane was placed on adherent cells grown on fibronectin-coated surface. The cells were then cleaved by lifting the membrane to obtain the VPM. The preparation was fixed and double stained (without detergent

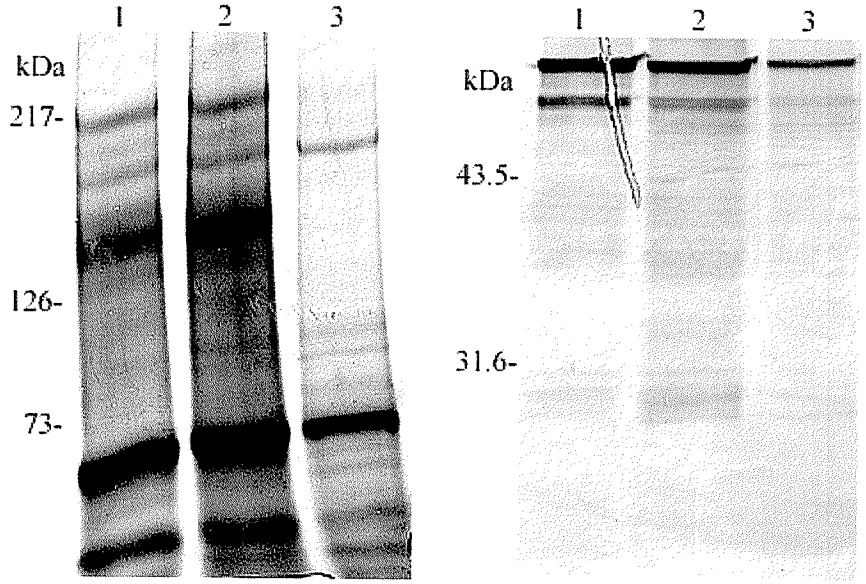

Fig. 3.2: Isolation of integrin associated molecules using the cytoplasmic domain of β1 integrin. β1 integrin captured with N29-beads was incubated with freshly made K562 cell-lysate.

Lane 1: β1 integrin-N29-beads incubated with lysis buffer (integrin control).

Lane 2: β1 integrin-N29-beads incubated with K562 cell lysate

Lane 3: N29-beads incubated with K562 cell lysate (lysate control).

permiabilization) for β1 integrin and paxillin/vinculin (fig. 3.3 and fig. 3.4). Paxillin and vinculin are two focal adhesion proteins that are known to colocalize with integrin-containing adhesion sites. Demonstration of their colocalization with integrin molecules in these experiments has proven that this protocol of VPM generation was gentle enough to preserve the adhesion structures. It should also be noted that the staining was performed without detergent permiabilization indicating the removal of dorsal membrane of the cells.

To isolate adhesion complexes for biochemical analysis, VPM preparations were made from cells grown on fibronectin-coated petridishes. The material thus prepared was dissolved in hot gel-loading buffer and resolved by SDS-PAGE under reducing conditions. To compare, an aliquot of whole cell lysate was run in the adjacent lane. The gel was subsequently silver stained and the protein bands were compared (fig.3.5). Contrary to expectations, there were no differences in the protein band pattern observed between the VPM-lane and cell-lysate lane. Each of the bands in the cell-lysate had a matching partner in the VPM-lane suggesting the impure nature of the isolated VPM, i.e., the isolated VPM preparation contained contaminated cellular proteins. It was obvious that the contaminants were from the leftover plasma membrane remnants in the VPM preparation. Subsequent efforts using lipase enzyme to remove the plasma membrane remnants while keeping the adhesion complexes intact were unsuccessful. These experiments suggested that the VPM preparation unsuitable for generating pure adhesion structures for biochemical

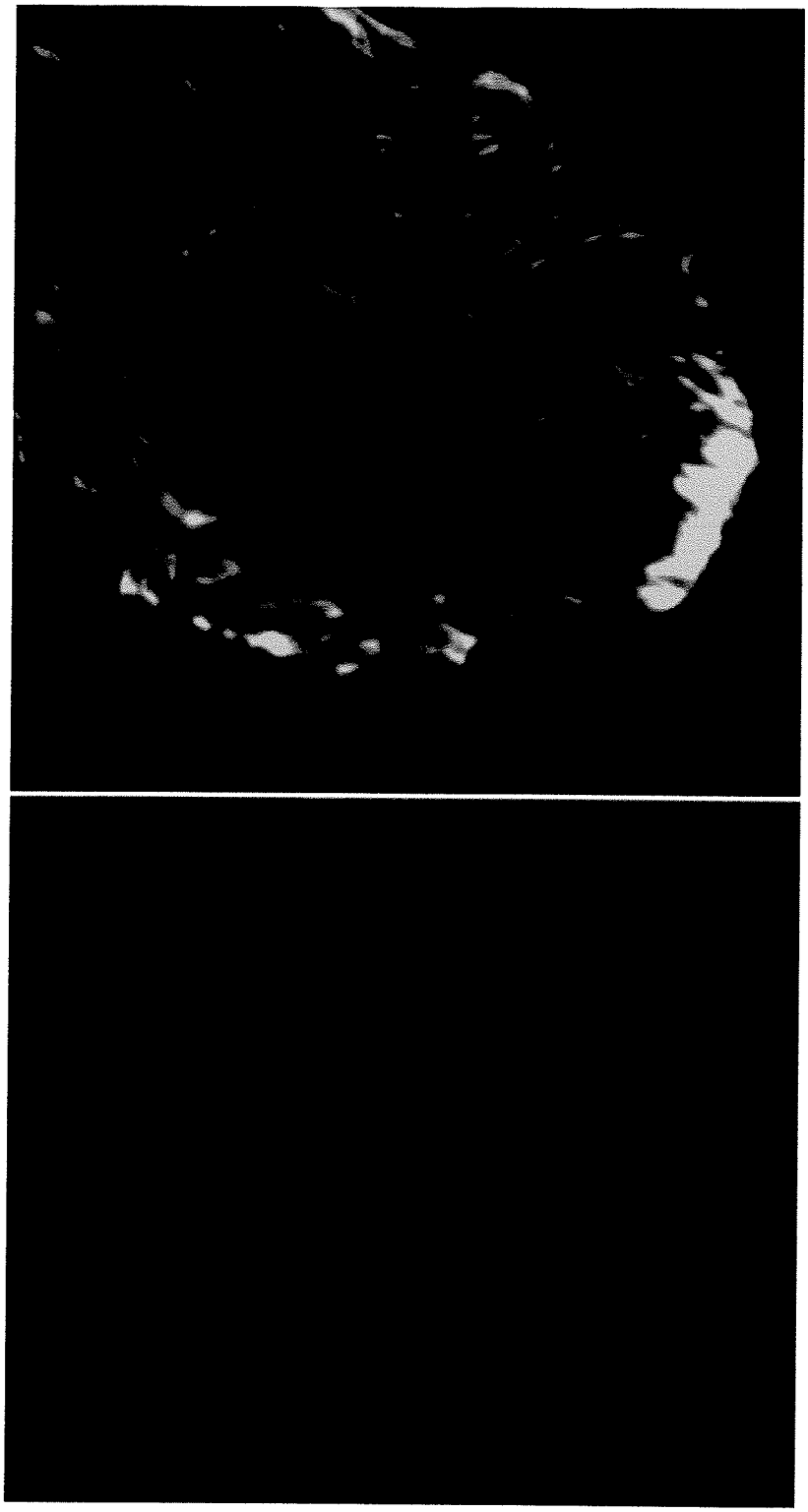

Fig. 3.3: Double staining of VPM prepared from human foreskin fibroblasts.

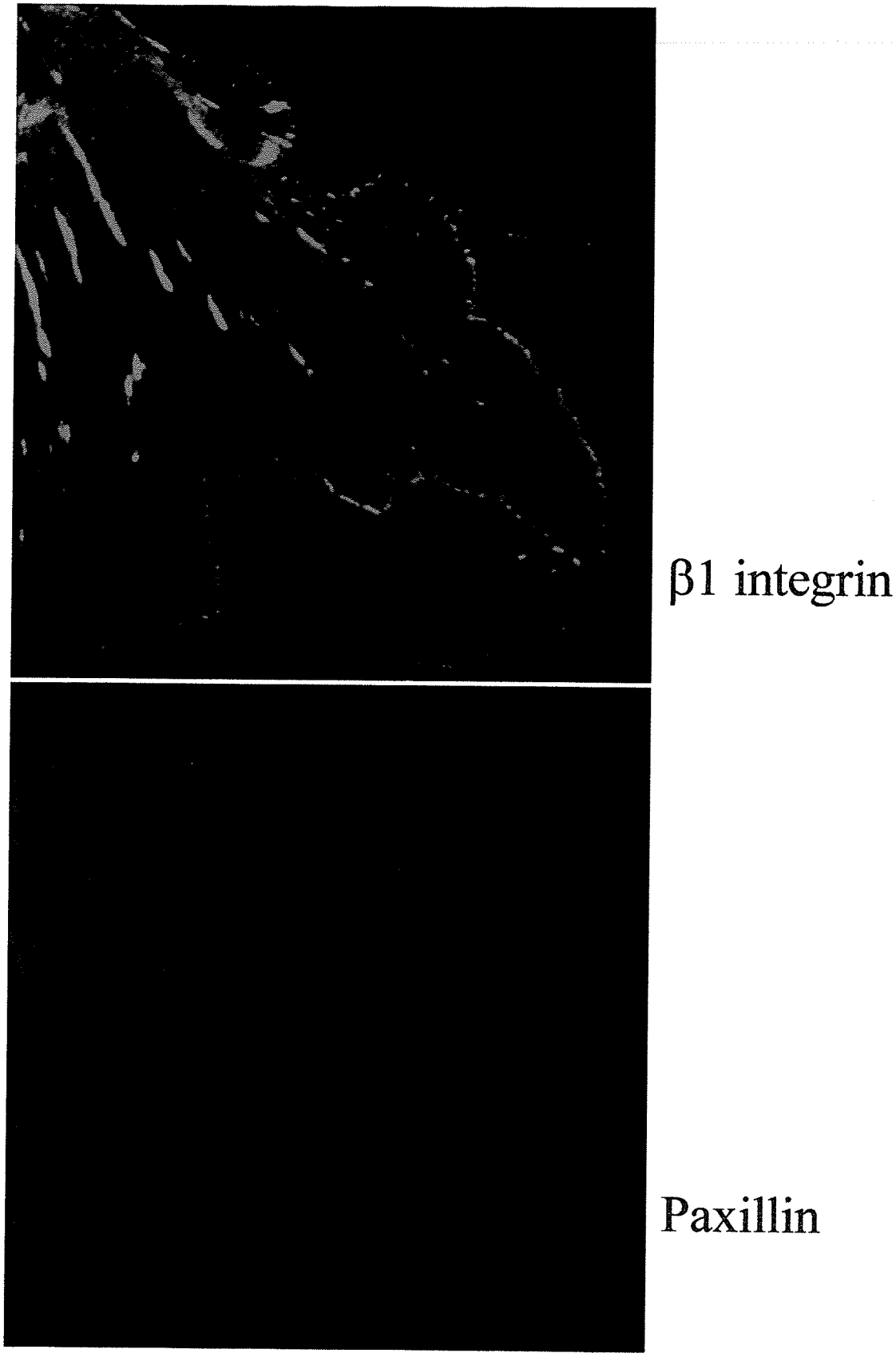

Fig. 3.4: Double staining of VPM prepared from human foreskin fibroblasts

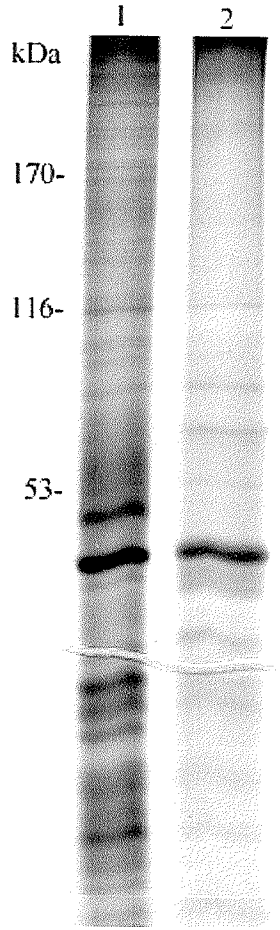

Fig. 3.5: Isolation of integrin associated molecules from VPM preparation. VPM preparation was dissolved in gel-loading buffer (reducing) and the protein molecules were resolved on a 10% SDS polyacrylamide gel (lane 1). For comparison, an aliquot of cell lysate was run along side (lane 2).

analysis. Therefore, a third approach, using anti-integrin antibody coated magnetic beads has been sought.

3.4. Induction and Isolation of Adhesion Complexes Using Antibody Coupled Magnetic Beads

In this approach, adhesion complexes were induced by incubating cells with anti-β1-integrin antibody coupled magnetic beads (Plopper and Ingber, 1993). The bound cells were lysed in cytoskeleton (CSK) buffer to isolate adhesion complexes stuck to the beads. CSK buffer has been shown to maintain the integrity of cytoskeleton and therefore this approach was thought to have the potential to isolate adhesion complexes intact. An aliquot of the preparation thus isolated was probed for some known focal adhesion proteins by immunoblotting (fig. 3.6). Talin, paxillin and vinculin are well-studied focal adhesion proteins that have been co-immunoprecipitated with β1 integrin. Detection of these molecules suggested that this complex-isolation protocol was suitable to preserve these supramolecular complexes. Moreover, their presence in 3S3-bead isolated material but not in the one isolated with anti CD3-beads, proved the specificity of this interaction. Having seen these results, it was decided to isolate adhesion complexes using this protocol and analyze them by mass spectrometry.

3.5. Identification of the Isolated Molecules by Mass Spectrometry

Adhesion complexes were isolated by incubating $3X10^7$ K562 cells with $3X10^8$ 3S3 coupled magnetic beads at 37°C for 30 minutes. A sample observed under

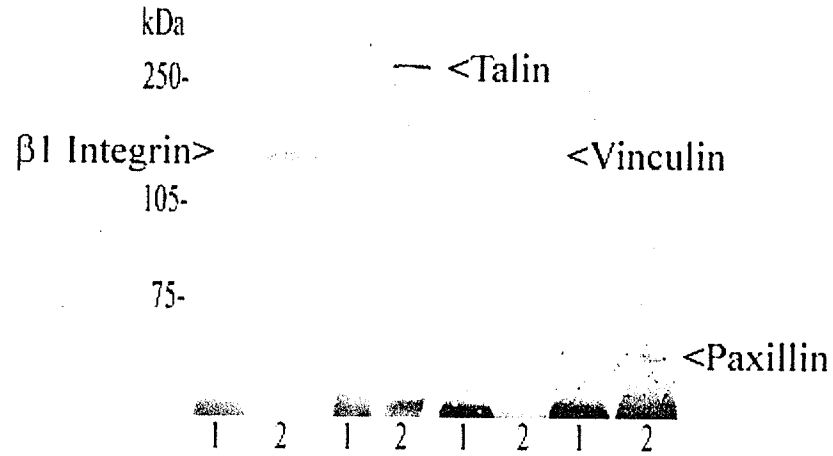

Fig. 3.6: Western blot analysis of an aliquot of adhesion complexes for some known focal adhesion proteins.

Lane 1: Material isolated with anti-CD3 beads,

Lane 2: Material isolated with 3S3 beads

microscopy at the end of incubation showed rosetting of cells with 3S3-beads but not with anti-CD3 coated beads (fig. 3.7), indicating the formation of cellular aggregation due to the specific interaction of integrin and anti integrin antibody.

The material bound to the beads after cell lysis was subsequently washed, dissolved in gel-loading buffer and resolved by SDS-PAGE under reducing conditions (fig 3.8). Even though there were differences in the intensities of protein bands between test and control lanes, a large number of non-specific bands were present. However, β1 integrin and three integrin-associated molecules were present only in the test but not in the control by western blot (fig. 3.6). Therefore, it was decided to excise as many number of protein bands as possible from the test lane to be identified by mass spectrometry. The specificity and validity of any molecule of interest thus identified was decided to determine by demonstrating its presence only in the 'test' but not in the 'control' by western blot analysis. For mass spectrometry analysis, as many protein bands as possible were excised under transillumination and subjected to in gel trypsin digestion as per materials and methods. The acquired mass spectrometry data were used to search the protein database to identify the molecules. To confirm the identity, a few peptides were selected from each mass spectrum and subjected to tandem mass spectrometry.

25 molecules were identified by the mass spectrometry analysis (Table 3.1). Surprisingly, neither β1 integrin nor any of the known integrin associated molecules were detected by mass spectrometry. One molecule, Ras**G**AP SH**3** domain-**b**inding **p**rotein (G3BP) was studied further because of the reasons stated below.

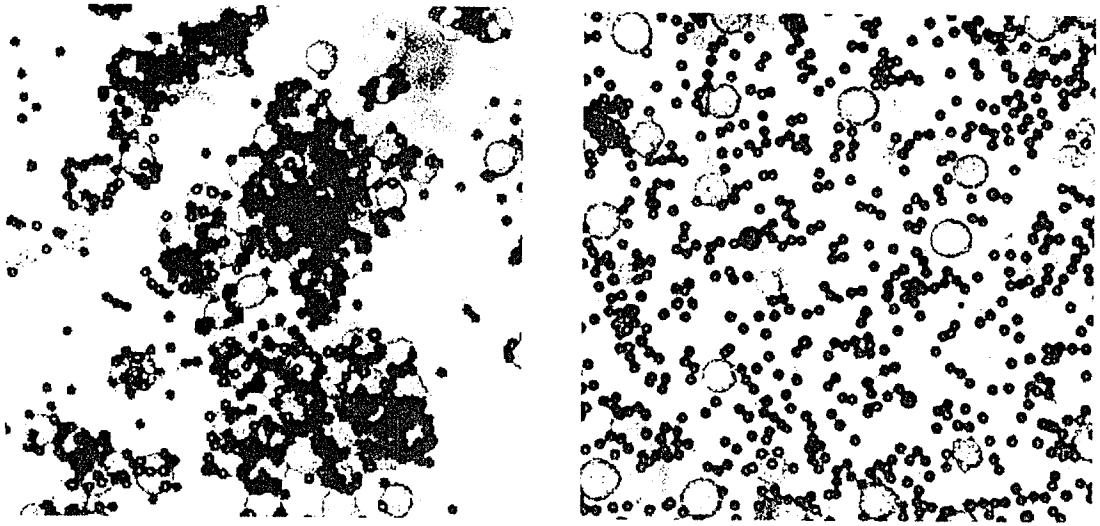

Fig. 3.7: Integrin-cross linking mediated cellular aggregation: K562 cells were incubated with 3S3-coupled magnetic beads to induce adhesion complexes formation. Anti-CD3 antibody coupled magnetic beads were used as negative control.

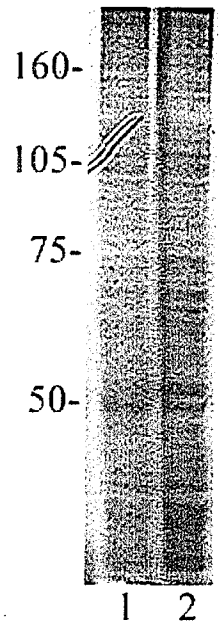

Fig. 3.8: Silver stained gel of adhesion complexes. Material isolated from $15X10^6$ cells using either 3S3-beads (lane: 2) or anti CD3-beads (lane:1) were resolved on a 8% SDS-PAGE under reducing conditions.

MS Identification	MS/MS Identification
Titin Zinc Finger protein	
No peaks	
p100 co-activator	**p100 co-activator**
PTB associated splicing factor	**PTB associated splicing factor**
HSP 90 Chaperone protein	
17-β hydroxy steroid dehydrogenase ABC transporter	**17-β hydroxy steroid dehydrogenase** **ABC transporter**
Poly A binding protein 70kDa heat shock protein	**Poly A binding protein** **70 kDa heat shock protein**
G3BP	**G3BP**
Nucleophosmin	**Nucleophosmin**
Human hypothetical protein	**Human hypothetical protein** **Unnamed protein**
αTubulin Mouse γ1 heavy chain	**αTubulin** Mouse γ1 heavy chain
Tubulin β chain Ig γ chain	**Tubulin β chain** Ig γ chain
Integrin α-E chain Zinc finger protein	**Ribosomal protein**
4 peaks	**hnRNP U**
Ribosomal protein L4	**Ribosomal protein L4**
Laminin-binding protein	**Laminin binding protein**
HnRNP protein X homolog	**HnRNP protein X homolog**
Ribosomal protein L4	**Ribosomal protein L4**
Acidic ribosomal protein	**Acidic ribosomal protein**
60S ribosomal protein L6	**60S ribosomal protein L6**
hnRNP A2/B1 60S ribosomal protein L5	**hnRNP A2/B1** **60S ribosomal protein L5**
Ribosomal protein S3	**Ribosomal protein S3**
Golgi membrane sialoglycoprotein	**Ribosomal protein**
Ribosomal protein S4	**Ribosomal protein S4**
Ribosomal protein L7	**Ribosomal protein L7**

Table 3.1: Proteins isolated from the adhesion complexes. The ones that are **bold** in the right column are of confirmed identity

1) G3BP's only known binding partner (at the time of this experiment) $RasGAP^{120}$ has been shown to influence cell polarity and cell migration, two of the known integrin mediated processes (Kulkarni et al, 2000).
2) $RasGAP^{120}$ regulates the activity of Ras, and Ras has been shown to enhance cell adhesion to extracellular matrix by activating integrin (Zhang et al, 1996).
3) G3BP homologue, *rasputin* knockout in developing Drosophila embryo interfered with the photoreceptor recruitment and ommatidial polarity in the eye (Pazman et al, 2000).

3.6. Mass Spectrometric Identification of G3BP

Band number 09 that appeared at about 65-70 kDa in the gel, gave 27 peptide masses upon MS analysis (fig. 3.9). These masses were used to search the protein database. Six of the masses (shown in figure 3.9) were identified as G3BP peptides (fig. 3.10). It was ranked first with a MOWSE score of 2.05e+004.

MOWSE score is based on the scoring system described by Pappin et al (1993). It is an acronym for **M**olecular **W**eight **S**earch and is one of the first algorithms developed to identify proteins by peptide mass fingerprinting. MOWSE scoring was originally created from the OWL non-redundant protein sequence database. The proteins in the database undergo theoretical digestion with the enzyme used in the experiment. The resulting peptides are then placed in bins based on their molecular weight and the intact molecular weight of undigested protein they originate from. Each calculated mass value which falls within a given mass tolerance of an

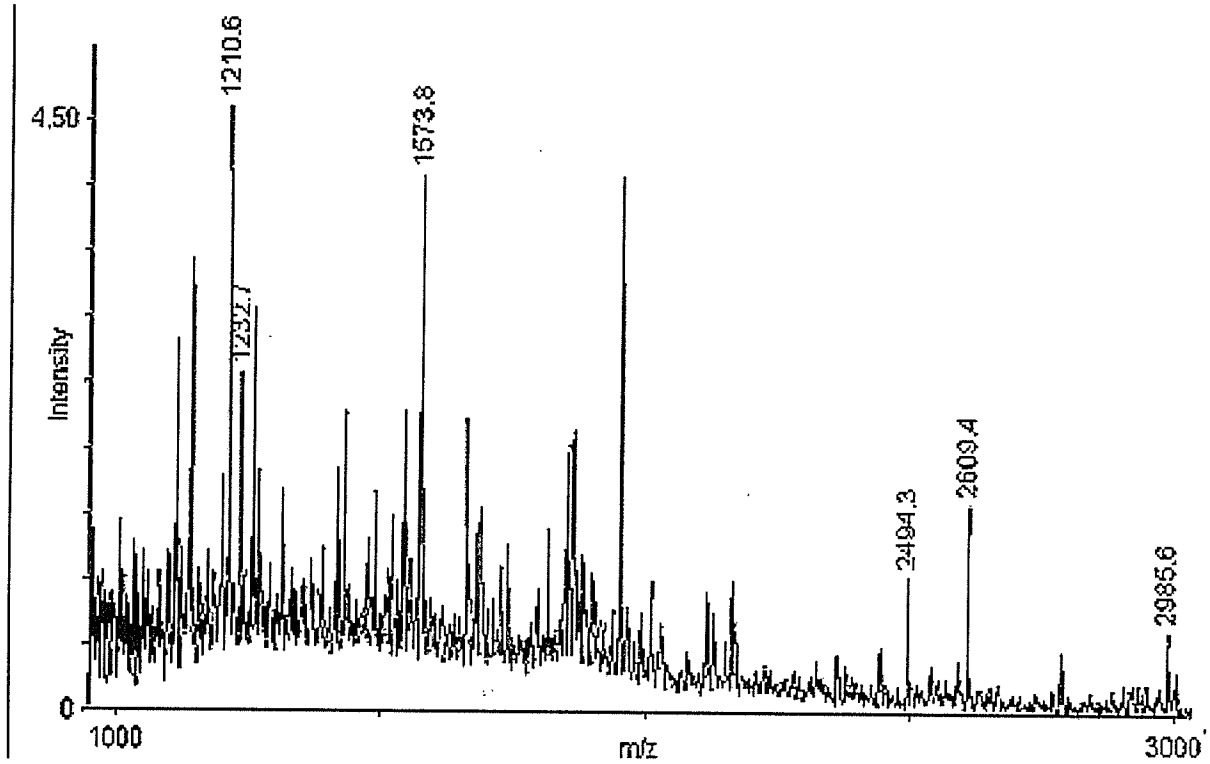

Fig. 3.9: Peptide finger print of band #09. Band#09 excised from the gel was trypsin digested and subjected to single-mass spectrometry. The spectrum had twenty seven peak masses and were used to search the NCBInr protein database.

MS-Fit Search Results

Press stop on your browser if you wish to abort this MS-Fit search prematurely.

Sample ID (comment): **sample09**
Database searched: **NCBInr.8.28.2000**
Full Molecular Weight range: **541351** entries.
Full pI range: **541351** entries.
Species search (**MAMMALS**) selects **89585** entries.
MS-Fit search selects **124** entries (results displayed for top **10** matches).

Min. # Peptides to Match	Peptide Mass Tolerance (+/-)	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by	Peptide N terminus	Peptide C terminus	Input # Peptide Masses
4	**30.000 ppm**	**monoisotopic**	**Trypsin**	**1**	**carbamidomethylation**	**Hydrogen (H)**	**Free Acid (O H)**	**27**

Result Summary

Rank	MOWSE Score	# (%) Masses Matched	Protein MW (Da)/pI	Species	NCBInr.8.28.2000 Accession #	Protein Name
1	**2.05e+004**	**6/27 (22%)**	**52164.5 / 5.37**	HOMO SAPIENS	5031703	(U32519) GAP SH3 binding protein

Fig. 3.10: Mass spectrometric identification of G3BP. Twenty seven peak masses obtained from band #09 were used to search the NCBInr protein database. Six masses were identified as that of G3BP which covered 22% protein(G3BP).

experimental value counts as a match. Unmatched masses are ignored. The score for each matching mass is assigned as the normalized distribution frequency value. Rather than just counting the number of matching peptides, MOWSE uses empirically determined factors to assign a statistical weight to each individual peptide match. After searching the experimental mass values against calculated peptide masses, the score for each entry is calculated. Matches using mass values are handled on a probabilistic basis. The total score is the absolute probability that the observed match is a random event. Reporting probabilities directly can be ambiguous because a "high" score is a "low" probability. Therefore, the scores are reported as – $10*LOG_{10}(P)$, where P is the absolute probability. An event is commonly considered significant if it occurs at random with a frequency of <5%. Therefore, scores greater than 69 are ($p<0.05$) significant (www.matrixscience.com/help/scoring_ help.html). The MOWSE score for G3BP was 2.05e+004 (i.e., 20500) and ranked 1st.

To further confirm the identity, three peptides, 1210.6, 1232.7, and 1573.8 were subjected to tandem mass spectrometry. There were 32 ions generated from peptide 1210.6 (fig. 3.11). The masses of those 32 ions were input for the subsequent protein database search that confirmed the peptide as that of G3BP (fig. 3.12). Similar results were also obtained with the other two peptides (figs. 3.13, 3.14, 3.15 & 3.16).

3.7. Westernblot Identification of G3BP in Adhesion Complexes

To identify G3BP by a conventional antibody based technique, an aliquot of adhesion complexes was subjected to western blot analysis using a monoclonal

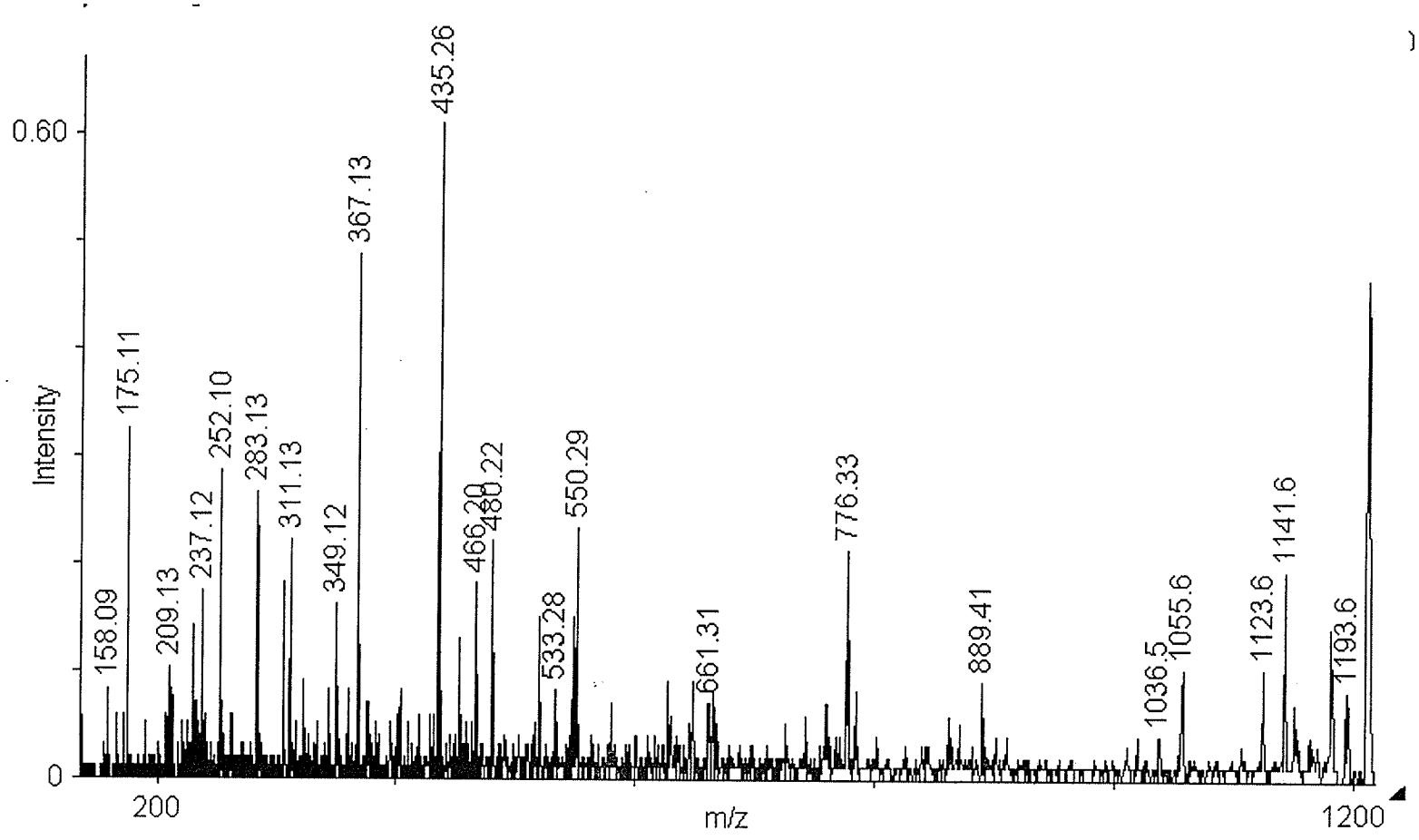

Fig. 3.11: Tandem mass spectrum of peptide 1210.6. Peptide 1210.6 was subjected to tandem mass spectrometry that generated 32 ions. These masses were used to search the NCBInr protein database

MS-Tag Search Results

Press stop on your browser if you wish to abort this MS-Tag search prematurely.

Sample ID (comment): **sample09, 1210.627**
Database searched: **NCBInr.8.28.2000**
Full Molecular Weight range: **541351** entries.
Full pI range: **541351** entries.
Species search (**MAMMALS**) selects **89585** entries.
Number of sequences passing through parent mass filter: **898**
MS-Tag search selects **2** entries.

Parent mass: **1210.6270 (+/- 30.0000 ppm)**
Fragment Ions used in search: **158.09, 175.11, 190.08, 207.08, 209.13, 212.11, 230.07, 233.16, 237.12, 252.10, 283.13, 305.15, 311.13, 349.12, 367.13, 435.26, 466.20, 480.22, 519.26, 547.28, 550.29, 627.31, 647.31, 660.26, 664.34, 776.33, 889.41, 1036.46, 1055.61, 1123.61, 1141.62, 1193.56 (+/- 50.00 ppm)**
Composition Ions present: **HFY**

Ion Types Considered: **a-NH3 a b b-NH3 b-H2O b+H2O y y-NH3 y-H2O I**

Search Mode	Max. # Unmatched Ions	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by	Peptide N terminus	Peptide C terminus
identity	**21**	**monoisotopic**	**Trypsin**	**1**	**carbamidomethylation**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH+ Calculated (Da)	MH+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.8.28.2000 Accession #	Protein Name
1	12/32	(K)FYVHNDIFR(Y)	1210.6009	0.0261	51829.0 / 5.41	MUS MUSCULUS	7305075	(AB001927) ras-GTPase-activating protein SH3-domain binding protein
1	12/32	(K)FYVHNDIFR(Y)	1210.6009	0.0261	52164.5 / 5.37	HOMO SAPIENS	5031703	(U32519) GAP SH3 binding protein

Fig. 3.12: Tandem mass spectrometric identification of G3BP. Masses of 32 daughter ions obtained from peptide 1210.6 by MS/MS were input to search the protein database.

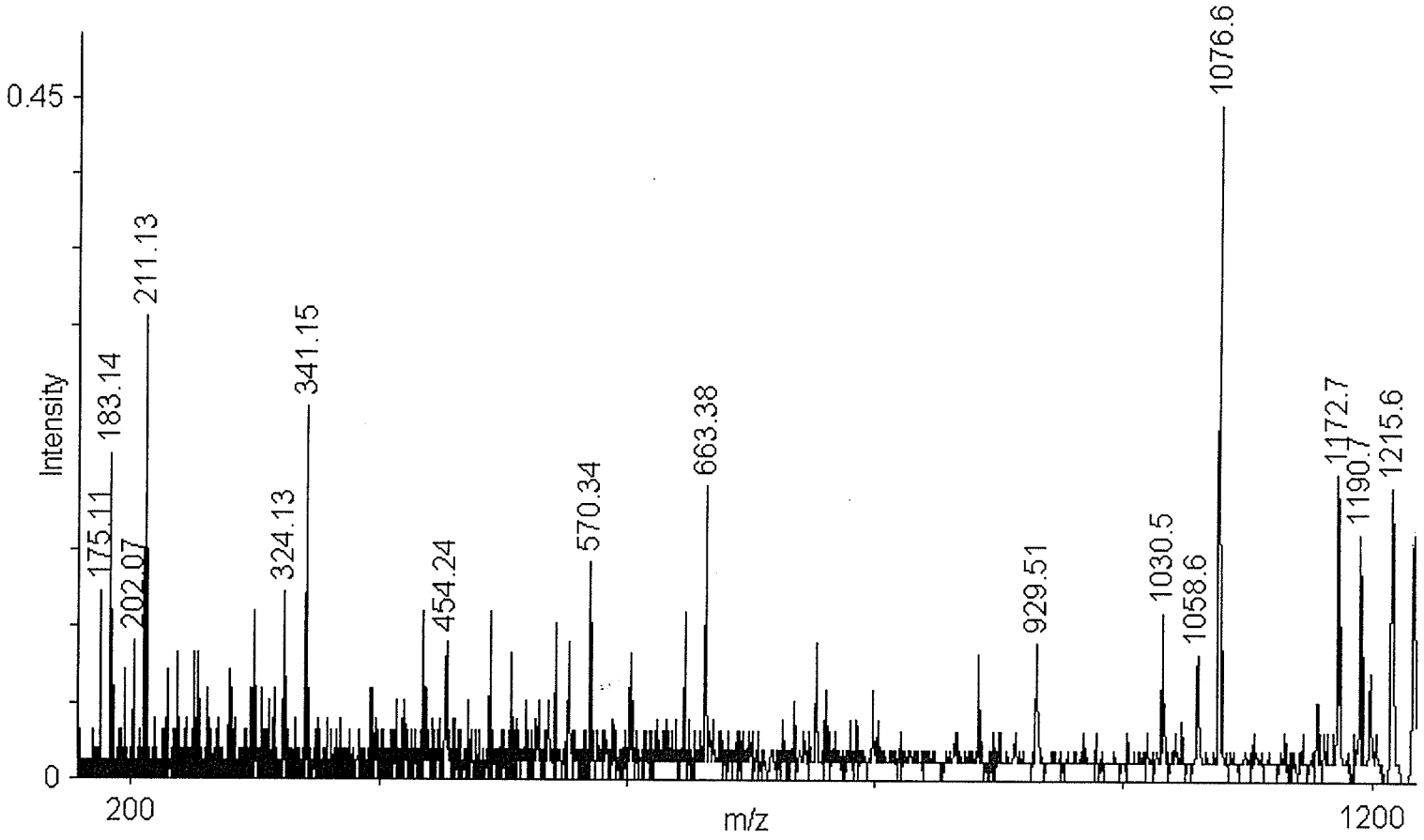

Fig. 3.13: Tandem mass spectrum of peptide 1232.7. Peptide 1237.7 was subjected to tandem mass spectrometry that generated 28 ions. These masses were used to search the NCBInr protein database

MS-Tag Search Results

Press stop on your browser if you wish to abort this MS-Tag search prematurely.

Sample ID (comment): **sample09, 1232.704**
Database searched: **NCBInr.8.28.2000**
Full Molecular Weight range: **541351** entries.
Full pI range: **541351** entries.
Species search (**MAMMALS**) selects **89585** entries.
Number of sequences passing through parent mass filter: **648**
MS-Tag search selects **2** entries.

Parent mass: **1232.7040 (+/- 30.0000 ppm)**
Fragment Ions used in search: **175.11, 183.14, 185.16, 202.07, 211.13, 213.15, 237.12, 300.18, 324.13, 341.15, 436.21, 454.24, 489.26, 542.34, 553.33, 570.34, 646.35, 663.38, 752.47, 883.52, 929.51, 1030.55, 1058.58, 1076.60, 1172.66, 1190.65, 1197.66, 1215.64 (+/- 50.00 ppm)**
Composition Ions present: **F**

Ion Types Considered: **a-NH3 a b b-NH3 b-H2O b+H2O y y-NH3 y-H2O I**

Search Mode	Max. # Unmatched Ions	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by	Peptide N terminus	Peptide C terminus
identity	**15**	**monoisotopic**	**Trypsin**	**1**	**carbamidomethylation**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH+ Calculated (Da)	MH+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.8.28.2000 Accession #	Protein Name
1	8/28	(K)VLSNRPIMFR(G)	1232.6938	0.0102	51829.0 / 5.41	MUS MUSCULUS	7305075	(AB001927) ras-GTPase-activating protein SH3-domain binding protein
1	8/28	(K)VLSNRPIMFR(G)	1232.6938	0.0102	52164.5 / 5.37	HOMO SAPIENS	5031703	(U32519) GAP SH3 binding protein

Fig. 3.14: Tandem mass spectrometric identification of G3BP. Masses of 28 daughter ions obtained from peptide 1210.6 by MS/MS were input to search the protein database.

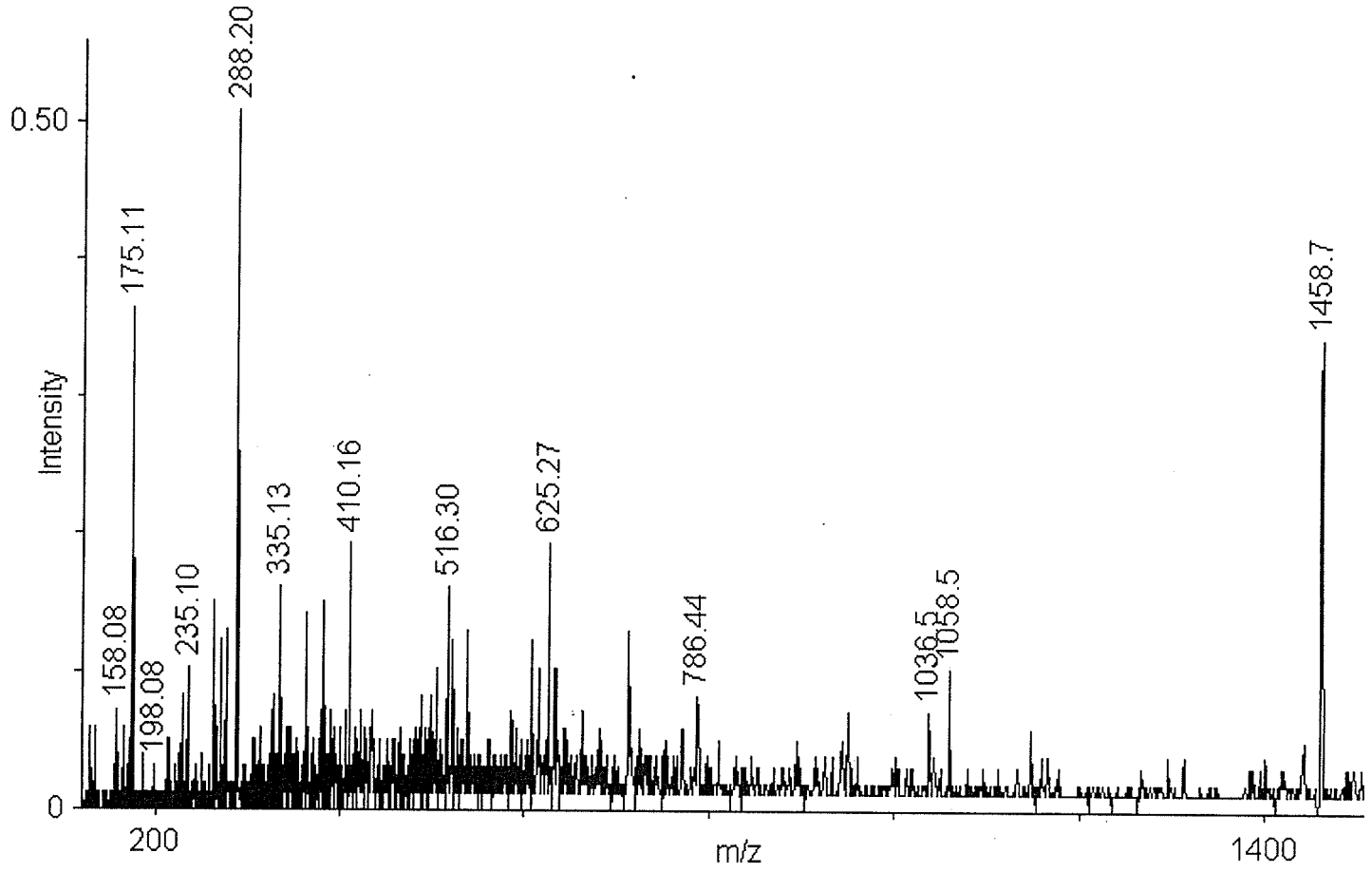

Fig. 3.15: Tandem mass spectrum of peptide 1573.8 . Peptide 1573.8 was subjected to tandem mass spectrometry that generated 27 ions. These masses were used to search the NCBInr protein database

MS-Tag Search Results

Press stop on your browser if you wish to abort this MS-Tag search prematurely.

Sample ID (comment): **sample09, 1573.807**
Database searched: **NCBInr.8.28.2000**
Full Molecular Weight range: **541351** entries.
Full pI range: **541351** entries.
Species search (**MAMMALS**) selects **89585** entries.
Number of sequences passing through parent mass filter: **785**
MS-Tag search selects **1** entry.

Parent mass: **1573.8070 (+/- 30.0000 ppm)**
Fragment Ions used in search: **158.08, 175.11, 198.08, 229.11, 235.10, 263.09, 271.16, 276.13, 288.20, 335.13, 363.16, 382.19, 410.16, 516.30, 521.24, 538.22, 607.26, 625.27, 632.29, 712.39, 949.51, 1036.53, 1058.46, 1147.56, 1458.72, 1555.74 (+/- 50.00 ppm)**
Composition Ions present: **FY**

Ion Types Considered: **a-NH3 a b b-NH3 b-H2O b+H2O y y-NH3 y-H2O I**

Search Mode	Max. # Unmatched Ions	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by	Peptide N terminus	Peptide C terminus
identity	**10**	**monoisotopic**	**Trypsin**	**1**	**carbamidomethylation**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH+ Calculated (Da)	MH+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.8.28.2000 Accession #	Protein Name
1	4/27	(K)DFFQSYGNVVELR(I)	1573.7651	0.0419	52164.5 / 5.37	HOMO SAPIENS	5031703	(U32519) GAP SH3 binding protein

Fig. 3.16: Tandem mass spectrometric identification of G3BP. Masses of 27 daughter ions obtained from peptide 1210.6 by MS/MS were input to search the protein database.

antibody to G3BP (fig. 3.17). Material isolated using anti-CD3 antibody coated beads did not contain G3BP suggesting its specific presence in adhesion complexes should β1 integrin was also present only in the material isolated using 3S3-beads but not anti-CD3 coupled beads.

3.8. Determination of G3BP1 Vs G3BP2

There are two RasGAP SH3 domain-binding proteins described, G3BP1 (or simply G3BP) and G3BP2. G3BP2 has two isoforms, A and B produced by alternate splicing (isoform B lacks residues 243-275). To determine the type of G3BP in the adhesion complexes, amino acid sequences of both proteins were retrieved from SWISS-PROT database (accession numbers Q13283 and Q9UN86 for G3BP1 and G3BP2 respectively) and were aligned using *clustal* program at EMBL (http://www.ebi.ac.uk/clustalw/). The three peptides (1210.6, 1232.7, and 1573.8), whose sequences were confirmed by tandem mass spectrometry, were found only in G3BP1 but not in G3BP2. To determine the peptide masses of sequences from the same region of G3BP2, *PeptideMass*, a peptide mass calculation tool at the Expasy website (http://ca.expasy.org/tools/peptide-mass.html) was used. Masses 1228.6, 1201.7 and 1574.8 were resulted (fig. 3.18) from G3BP2 instead of masses 1210.6, 1232.7, and 1573.8 of G3BP. All these data were put together in figure (3.19). To further confirm, G3BP2 sequences were subjected to *in silico* trypsin digestion using *Ms-Digest*, a protein digestion tool from the UCSF Mass Spectrometry Facility

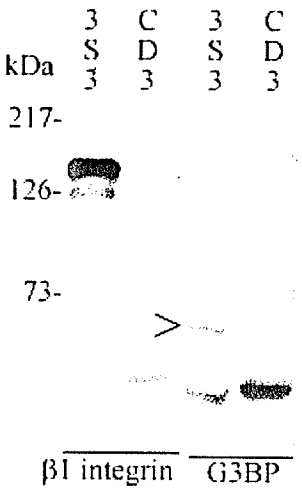

Fig. 3.17: Westernblot identification of G3BP in focal adhesion complexes. Focal adhesion complexes were prepared from $6X10^6$ HFF cells using $120X10^6$ 3S3-magnetic beads. Complexes from $1X10^6$ and $5X10^6$ cells were analyzed for integrin and G3BP respectively. Anti-CD3 antibody coupled beads were used instead of 3S3-beads as negative control.

Peptide Mass

mass	position	#MC	artif.modification(s)	peptide sequence
1228.557	1-9	0		FYVHNDMFR

mass	position	#MC	artif.modification(s)	peptide sequence
1201.749	2-11	0		ILIAKPIMFR

mass	position	#MC	artif.modification(s)	peptide sequence
1574.767	1-13	0		EFFMSFGNVVELR

Fig. 3.18: Masses of three G3BP2 peptide sequences, whose positions are corresponding to that of three G3BP peptides, 1210.6, 1232.7, and 1573.8

```
                    CLUSTAL W (1.81) multiple sequence alignment

XP_003842: RasGAP SH3-domain-binding protein  [Homo sapiens] 466 aa
AAD51932:  RNA-binding protein isoform G3BP-2a [Homo sapiens]482 aa


XP_003842       MVMEKPSPLLVGREFVRQYYTLLNQAPDMLHRFYGKNSSYVHGGLDSNGKPADAVYGQKE 60
AAD51932        MVMEKPSPLLVGREFVRQYYTLLNKAPEYLHRFYGRNSSYVHGGVDASGKPQEAVYGQND 60
                ************************:**: ******:********:*:.*** :******::

XP_003842       IHRKVMSQNFTNCHTKIRHVDAHATLNDGVVVQVMGLLSNNNQALRRFMQTFVLAPEGSV 120
AAD51932        IHHKVLSLNFSECHTKIRHVDAHATLSDGVVVQVMGLLSNSGQPERKFMQTFVLAPEGSV 120
                **:**:* **::***************.*************..*. *:**************

                    1210.6 (1228.6)
XP_003842       ANKFYVHNDIFRYQDEVFGGFVTEPQEESEEEVEE-PEERQQTPEVVPDDSGT-FYDQAV 178
AAD51932        PNKFYVHNDMFRYEDEVFGDSEPELDEESEDEVEEEQEERQPSPEPVQENANSGYYEAHP 180
                .********:***:*****.  .* :****:****  **** :** * :::.: :*:

XP_003842       VSNDMEEHLEEPVAEPEPDPEPEPEQEPVSEIQEEKPEPVLEETAPEDAQKSSSPAPADI 238
AAD51932        VTNGIEEPLEESSHEPEPEPESETKTEELKPQVEEKNLEELEE-------KSTTPPPAEP 233
                *:*.:** ***.  ****:**.*.: * :.   ***    ***       **::*.**:

XP_003842       AQTVQEDLRTFSWASVTSKNLPPSGAVPVTGIPPHVVKVPASQPRPESKPESQIPPQRPQ 298
AAD51932        VSLPQEPPKAFSWASVTSKNLPPSGTVSSSGIPSH-VKAPVSQPRVEAKPEVQSQPPR-V 291
                ..  **  ::****************:*. :***.* **.*.**** *:*** *  * *

XP_003842       RDQRVREQRINIPPQRGPRPIREAGEQGDIEPRRMVRHPDSHQLFIGNLPHEVDKSELKD 358
AAD51932        REQRPRE-RPGFPP-RGPRPGRGDMEQNDSDNRRIIRYPDSHQLFVGNLPHDIDENELKE 349
                *:** ** * .:** ***** *   **.* : **::*:*******:*****::*:.***:

                   1573.8 (1574.8)                    1232.7 (1201.7)
XP_003842       FFQSYGNVVELRINS---GGKLPNFGFVVFDDSEPVQKVLSNRPIMFRGEVRLNVEEKKT 415
AAD51932        FFMSFGNVVELRINTKGVGGKLPNFGFVVFDDSEPVQRILIAKPIMFRGEVRLNVEEKKT 409
                ** *:**********:   ********************::*  :*****************

XP_003842       RAARE-------GDRRDNR--LRGPGGPRGGLGGGMRG-------PPRGGMVQKPGFGVG 459
AAD51932        RAARERETRGGGDDRRDIRRNDRGPGGPRGIVGGGMMRDRDGRGPPPRGGMAQKLGSGRG 469
                *****       .**** *   ******** :****         ******.** * * *

XP_003842       RGLAPRQ------ 466
AAD51932        TGQMEGRFTGQRR 482
                 *    :
```

Fig. 3.19: Amino acid sequence alignment of G3BP1 & 2. All the three peptides (1210.6, 1573.8, 1232.7) analysed by tandem mass spectrometry were identified as derived from G3BP1but not G3BP2. Masses in parenthesis are the ones that would have resulted if the peptides were from G3BP2.

(http://prospector.ucsf.edu/ ucsfhtml3.4/msdigest.htm). None of the of the resulting peptides had the masses 1210.6, 1232.7, and 1573.8 (fig.3.20).

3.9. Integrin Clustering is Required for the Recruitment of G3BP

G3BP is a cytosolic protein and to test whether it is constitutively present in the adhesion complex or translocated to it upon integrin clustering, a cell lysate prepared in CSK buffer was incubated with 3S3-coupled magnetic beads and subsequently analyzed by immunoblotting for G3BP (fig. 3.21). Only β1 integrin was detected but not G3BP, suggesting the requirement of integrin clustering for G3BP's recruitment on to adhesion complexes.

3.10. Serum-Independent Recruitment of G3BP

It has previously been shown that G3BP, a cytosolic protein gets translocated to the membrane up on serum-starvation of the cells and under serum-fed conditions it is not demonstrable in the cell membrane (Gallouzi et al, 1998). To see if G3BP's recruitment to adhesion complexes is also dependent on the presence or absence of serum in the culture, adhesion complexes were isolated from serum-fed and 30 hours serum-starved cells and analyzed by immunoblotting. G3BP was present in both preparations suggesting its serum independent recruitment to adhesion complexes (fig.3.22).

3.11. Microscopic Demonstration of G3BP Localisation in Adhesion Complexes

To visualize G3BP in adhesion complexes, HFF cells were incubated with 3S3 or PLL-coupled latex beads and then layered on PLL coated coverslips. The cells

MS-Digest Results

Protein Name: **RAS-GTPASE-ACTIVATING PROTEIN BINDING PROTEIN 2 (GAP SH3-DOMAIN BINDING PROTEIN 2) (G3BP-2)**
Species: **HUMAN**
SwissProt.9.2.2001 Accession #: **Q9UN86**
MS-Digest Index #: **99212**
pI of Protein: **5.41**
Protein MW: **54111.5** Da
Amino Acid Composition: **A17 C1 D23 E58 F19 G46 H13 I13 K22 L26 M12 N21 P48 Q21 R42 S34 T16 V38 W1 Y11**

Start	End	m/z (mi)	Missed Cleavages	Sequence
217	224	1003.4948	0	(K) NLEELEEK (S)
18	25	1042.5573	0	(R) QYYTLLNK (A)
243	252	1083.5475	0	(K) AFSWASVTSK (N)
388	397	**1201.7495**	0	(R) ILIAKPIMFR (G)
124	132	**1228.5573**	0	(K) FYVHNDMFR (Y)
312	322	1280.4813	0	(R) GDMEQNDSDNR (R)
206	216	1329.6902	0	(K) TEELKPQVEEK (N)
65	76	1434.7051	0	(K) VLSLNFSECHTK (I)
1	13	1456.8020	0	(-) MVMEKPSPLLVGR (E)
278	290	1464.7811	0	(R) VEAKPEVQSQPPR (V)
349	361	**1574.7677**	0	(K) EFFMSFGNVVELR (I)
253	270	1763.9292	0	(K) NLPPSGTVSSSGIPSHVK (A)
108	123	1764.8995	0	(K) FMQTFVLAPEGSVPNK (F)
225	242	1871.9755	0	(K) STTPPPAEPVSLPQEPPK (A)
371	387	1965.9711	0	(K) LPNEGEWVEDDSEPVQR (I)

Fig. 3.20: *in silico* digestion of G3BP2. Masses of peptides that are closer to the three G3BP1 peptides (1210.6, 1232.7, and 1573.8) are shown in red

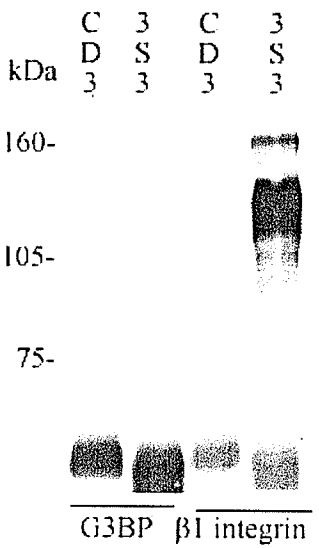

Fig. 3.21: Integrin clustering is a requirement for G3BP recruitment. $6X10^6$ HFF cells were lysed in CSK buffer and incubated with $120X10^6$ 3S3-magnetic beads. Material in the beads were tested for β1 integrin and G3BP by immunoblotting. Complexes from $1X10^6$ and $5X10^6$ cells were analyzed for integrin and G3BP respectively.

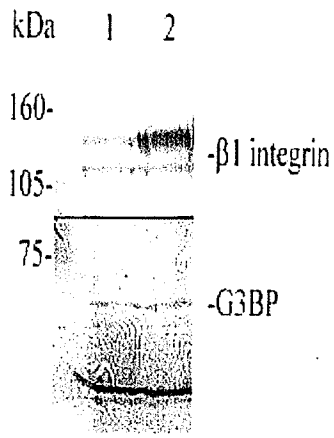

Fig. 3.22: Serum independent recruitment of G3BP.

Lane 1: Serum starved, Lane 2: Serum fed

were PFA fixed, detergent permiabilized and subsequently stained for G3BP or β1 integrin. Presence of G3BP was readily visible around the 3S3 beads but not around the PLL coated beads and was identical with the appearance of β1 integrin around the beads (fig. 3.23).

3.12. RasGAP^{120} is also Present in the Adhesion Complexes

The only known binding partner of G3BP (at the time of this experiment) was RasGAP^{120}. To determine if RasGAP^{120} also present in the adhesion complexes, an aliquot of adhesion complexes was probed for RasGAP^{120} by western blotting. It revealed its presence in adhesion complexes (fig.3.24), suggesting the possibility of G3BP recruitment along with RasGAP^{120}.

3.13. Functional relevance of G3BP in Adhesion Complexes

Loss of *rasputin*, Drosophila homologue of G3BP (Pazman et al, 2000) or RasGAP^{120}, G3BP's binding partner have been shown to interfere with cell polarity and cell migration (Kulkarni et al, 2000). To determine if G3BP also had a similar effect, cell-wound assay was performed on a confluent layer of HFF cells. To visualize the entire cell migrating towards the wound, especially the leading edge (lamellipodium and filopodium), phalloidin-FITC was used along with G3BP staining. Filopodial structures contained G3BP close to the leading edge (figs. 3.25). However G3BP was excluded from the lamellipodial structures (fig. 3. 26).

To test whether G3BP's translocation towards the cell periphery was time-dependent, HFF cells were allowed to adhere and briefly spread on fibronectin-coated

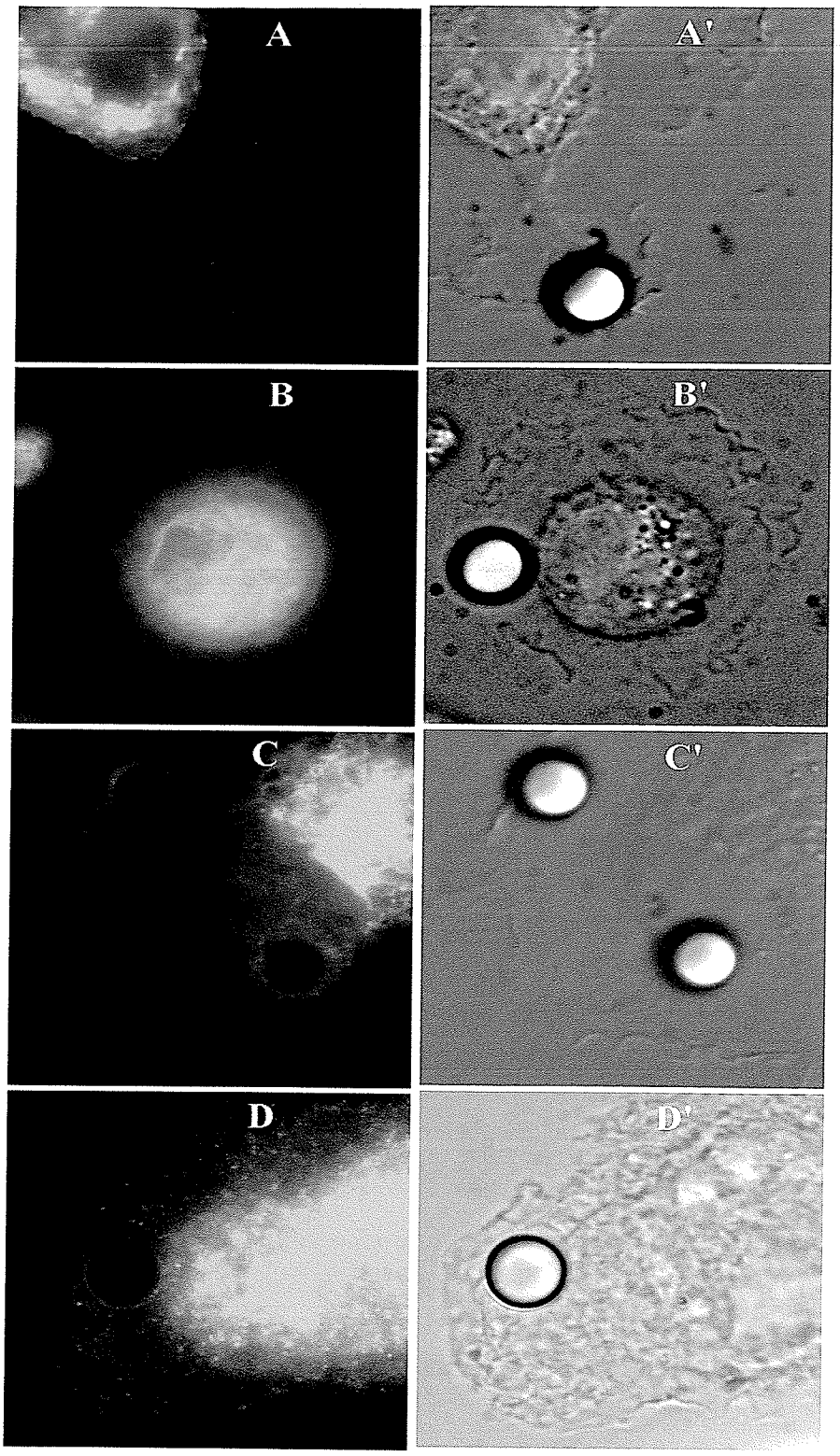

Fig. 3.23: Localization of G3BP at the adhesion sites.
A&B: β1-integrin, C&D: G3BP, A&C: 3S3-bead, B&D: PLL-bead
A'- D': intreference reflection images

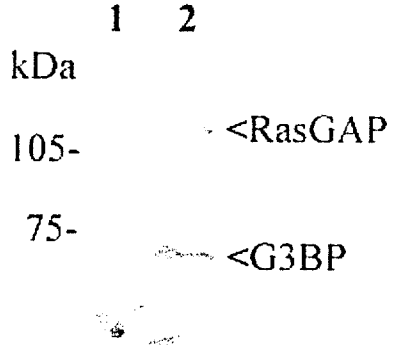

Fig. 3.24: Recruitment of RasGAP^{120} to adhesion complexes. Adhesion complexes prepared from 8X10^{6} HFF cells were probed for the presence of RasGAP^{120} by immunoblot.

Lane 1: Anti CD3-bead isolated material

Lane 2: 3S3-bead isolated material

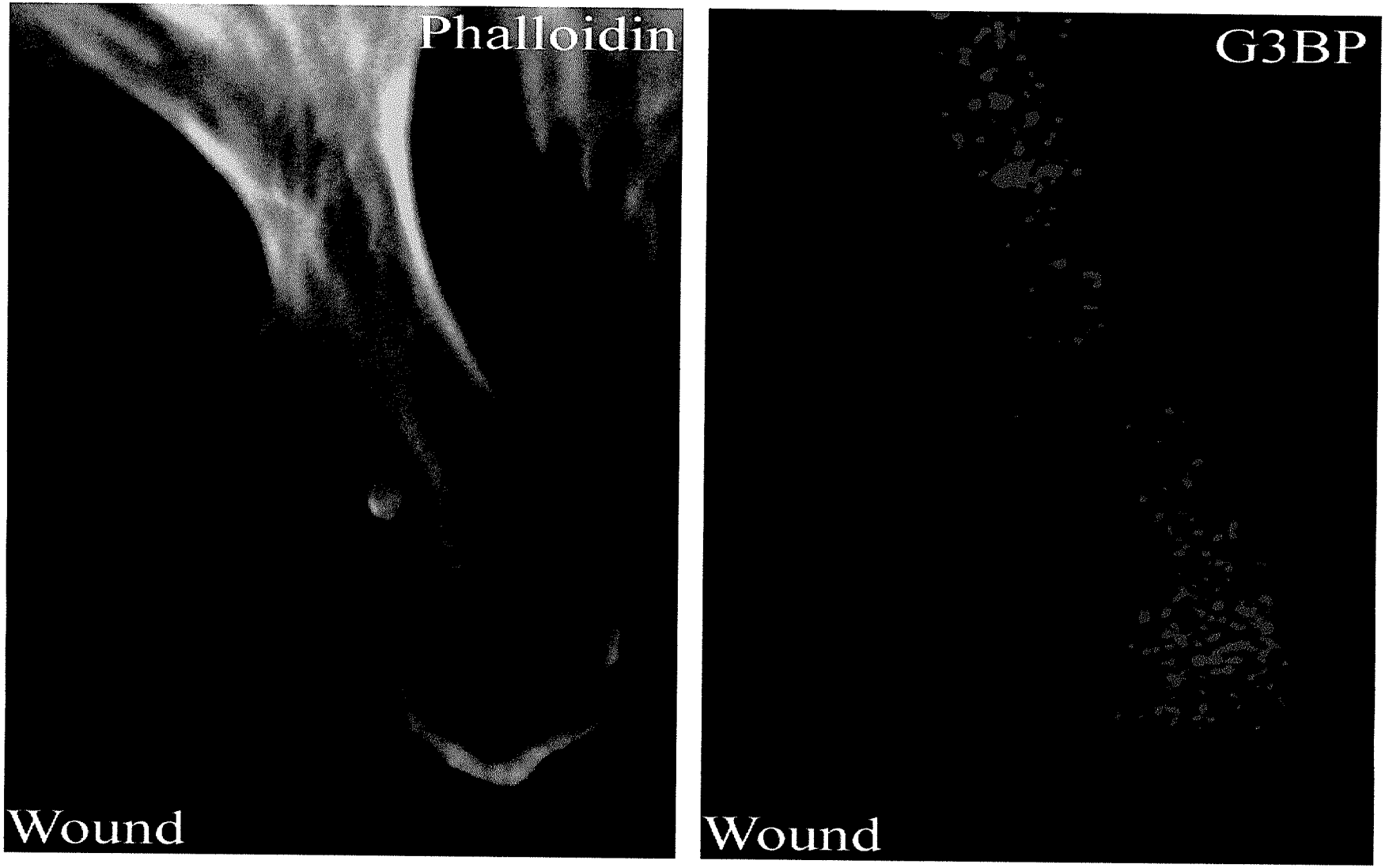

Fig. 3.25: Cell-wound Assay. G3BP and phalloidin double staining showing G3BP in filopodial structure of a migrating cell

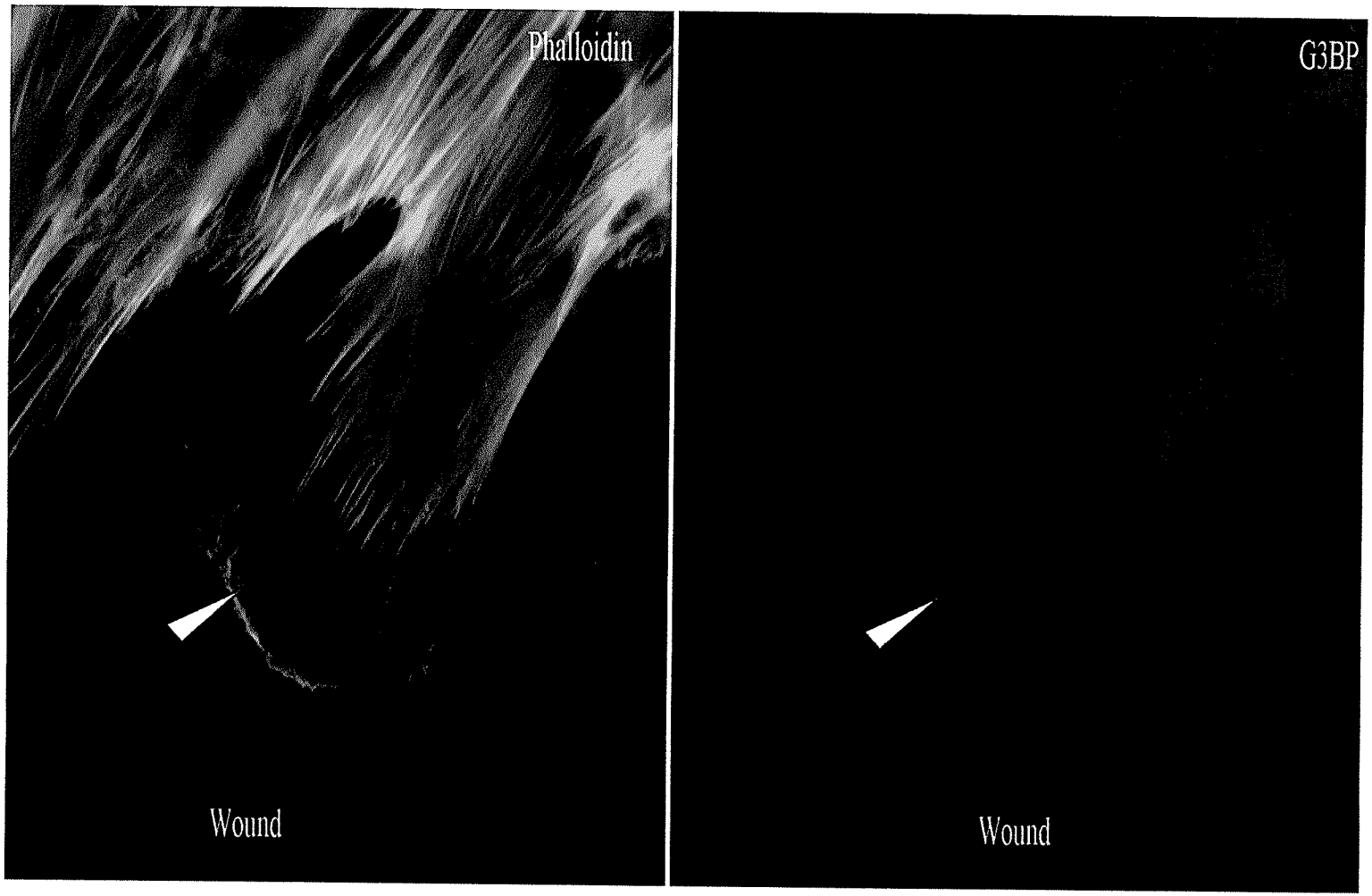

Fig. 3.26: Cell-wound Assay. G3BP and phalloidin double staining showing lamellipodium of a cell migrating towards the wound with G3BP excluded (arrow)

coverslips before doubly staining with phalloidin and G3BP. It appeared that the cells during the early stage of adhesion and spreading retained G3BP within the cortical actin (fig. 3.27). However, later in the process of spreading the cortical actin became less pronounced and G3BP starts appearing at the spreading edges of the cell (fig. 3.28). Thus it appears that the accumulation of G3BP on to the spreading edges of the cells is temporal.

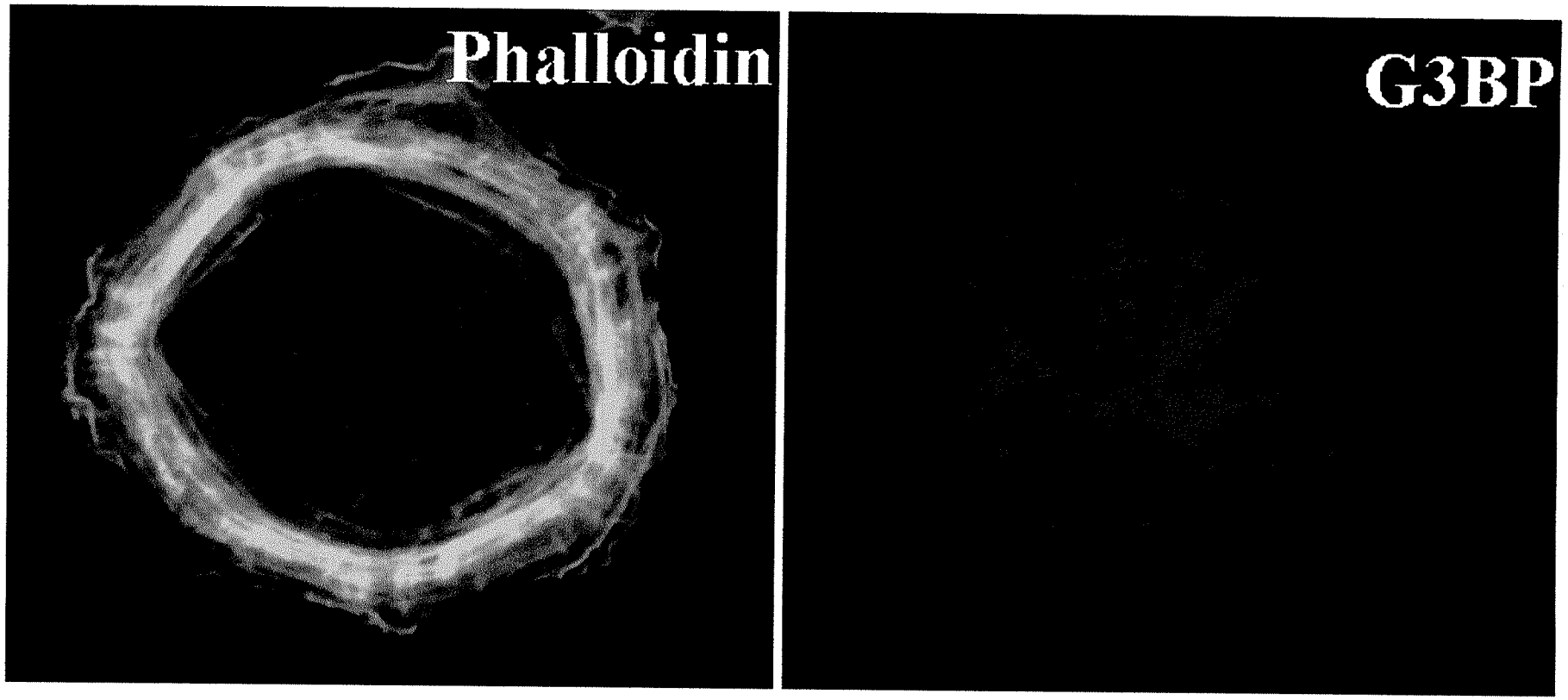

Fig. 3.27: G3BP and phalloidin double staining. G3BP is retained within the cortical actin of a cell beginning to spread

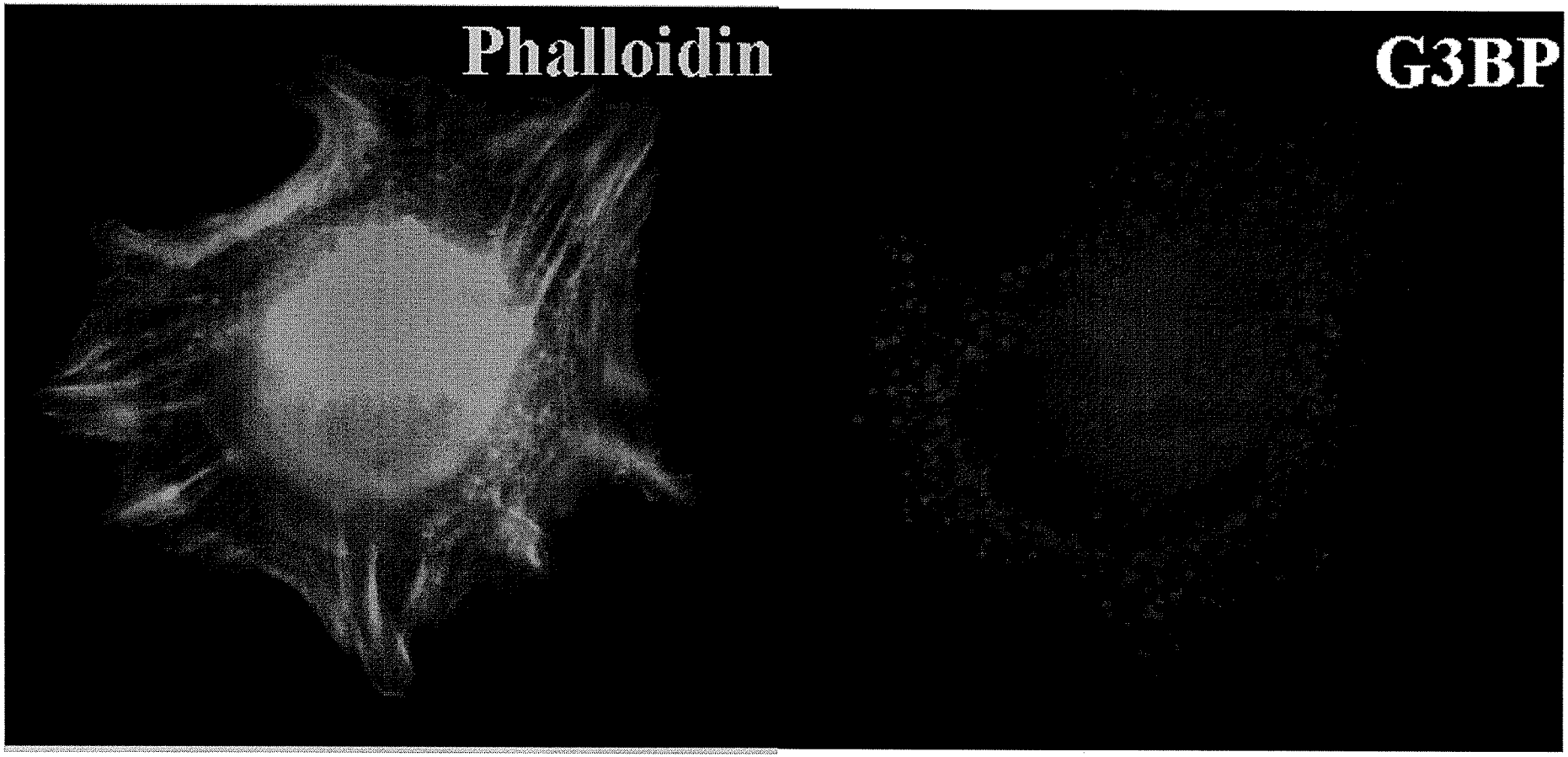

Fig. 3.28: G3BP and phalloidin double staining. A spreading cell has G3BP as far as its outer edges

4. DISCUSSION

The objective of this project was to isolate the integrin containing adhesion complexes intact and identify their true molecular composition using mass spectrometry analysis. Understanding the molecular composition and stoichiometry of these supromolecular complexes would lead us to understand the adhesion process. Three different methods were attempted to isolate the adhesion complexes intact. One of the methods identified a molecule, G3BP, in adhesion complexes that was previously unknown as part of adhesion complexes. Identification of G3BP in adhesion complexes wouldn't have been possible without mass spectrometry based protein identification.

Some of the molecules in the adhesion complexes may interact weakly, transiently and/or exist in minute quantities, making it extremely difficult to isolate adhesion complexes intact. These together with the enormous number of non-specifically bound proteins failed to provide pure adhesion complexes by all the three methods, which are discussed in detail below.

4.1. Fishing Approach

What are the possible reasons for the failure of this approach

1) The integrin used in this experiment was functional by its ability to bind to one of the ligands, fibronectin. However, it was a total human β1 integrin preparation, exact integrin species and the type of β1 integrin isoforms present were unknown. It has been shown that β1B isoform negatively affect the function of β1A, the

ubiquitous β1 isoform (Balzac et al, 1994). Therefore, it is possible that the presence of this dominant negative isoform in the β1 preparation prevented other β1 isoforms from binding to focal adhesion protein molecules.

2) It is known that, integrin molecules upon ligand binding move laterally across the cell membrane and cluster (Kucik et al, 1996; Yauch et al, 1997). Integrin clustering is necessary for the accumulation of some cytoskeletal molecules (Miyamoto et al, 1995; Miyamoto et al, 1995). This leads to signaling that recruits the molecules to and associate with the cytoplasmic domain of integrin. In this experimental approach, the integrin molecules were not on the semi-fluid cell membrane, its normal habitat, but on solid substrate, the beads. This was the major drawback of this experimental approach. But in an elegant study, Goldmann (2000) has shown purified αIIbβ3 integrin binding directly to known focal adhesion proteins such as talin, filamin, f-actin and α-actinin. But he has used purified focal adhesion proteins instead of cell lysate. He has also demonstrated the binding affinity of these molecules with integrin as low (K_d between 0.4 μM to 5μM). Moreover, higher concentration of integrin and focal adhesion proteins (3μM) were required for them to interact. Therefore, the major exception of the current experiment with that of Goldmann's is that the concentration of reactants was much lower. His buffer salt concentration was also lower, 56 mM in comparison to 130 mM used in the current study. However, there were no differences in the protein bands between the test and controls in the experiment that I have performed. Failure of this approach to pick up

any focal adhesion proteins could also be due to the limited resolving power of single-D gel electrophoresis.

4.2. VPM Approach

Ventral plasma membrane preparations were used in the past for various studies. For example, immunostaining of intracellular antigens on the cytoplasmic side of plasma membrane without perturbations by fixatives and detergents (Brands and Feltkamp, 1988), to study the assembly of clathrin coated pits on plasma membrane (Moore et al, 1987), to enrich plasma membrane proteins (Wang et al, 1999), and to study the transport of viral glycoproteins from the trans-golgi network to the basolateral plasma membrane (Mayer et al, 1996). VPM preparations thus, were proven to be excellent models to study membrane-bound structures from within the cell.

As for the integrin, VPM preparations have been used to study focal adhesion components and associated cytoskeleton (Anvur and Geiger, 1981; Gates et al, 1993; Cattelino et al, 1999). However, these studies were largely focused on the behavior of focal adhesion contacts and integrin molecules on the intact membrane, rather than isolating adhesion complexes for biochemical analysis. Anvur and Geiger used VPM preparations to study the effect of pH on the membrane contacts with the substrate. They have found at pH 7.0-7.2 the attachment was mediated predominantly by focal contacts, whereas at pH 6.0 the membrane reversibly formed extensive close contacts with the substrate (Avnur and Geiger, 1981). Cattelino et al, have used VPM to

explore the mechanisms regulating integrin distribution and function on the plasma membrane. They have reported high Ca^{++} concentrations induce quasi-reversible diffusion of β1 integrins out of focal adhesions, whereas low Ca^{++} concentrations induce irreversible recruitment of β1 receptors along extracellular matrix fibrils. Their further experiments with cells expressing truncated β1 integrin proved the requirement of cytoplasmic domain for low Ca^{++} induced recruitment of integrins to the matrix fibrils (Cattelino et al, 1999).

One major attraction of VPM preparation for isolating adhesion complexes intact was it did not involve detergent usage, and therefore expected to keep all the molecules together that are interacting weakly and/or hydrophobically. However, the experiments performed for this thesis failed to produce clean adhesion complexes for mass spectrometry analysis. It was because of the contaminant proteins from the plasma membrane remnants of VPM. Different dilutions of phospholipase enzyme treatment failed to remove the plasma membrane remnants while keeping the adhesion complexes intact. Therefore, it is logical to conclude that VPM preparation as unsuitable for adhesion complex isolation for mass spectrometry analysis. This preparation could be ideal for a system, for example *laser tweezers,* where individual focal adhesion complexes can be picked up and analyzed by mass spectrometry. But the current sensitivity range of mass spectrometry requires an impractical number of complexes to be picked up for such analysis. A subtraction/differential approach was another thing that could have been done, where the protein bands obtained from the

VPM preparation were compared with that derived from a preparation of plasma membrane.

4.3. Antibody Coupled Bead Approach

This approach was able to identify 25 molecules by mass spectrometry but failed to detect any of the known focal adhesion proteins including β1-integrin. β1-integrin and three known focal adhesion proteins were detected by immunoblotting in 3S3-beads isolated material but not in anti-CD3-beads isolated material. This shows that the mass spectrometry analysis failed to detect some molecules that were present in the complexes.

There were a large number of protein bands in the silver-stained gel. It appeared that most of them were non-specifically bound to the beads since material isolated with anti-CD3 coupled beads also contained an enormous number of bands. Because of the enormity of bands together with the limited resolving power of single-D gel, it was impossible to excise all individual protein bands separately for trypsin digestion. So, the multiple number of proteins in the gel piece may have undergone disproportionate trypsin digestion resulting in the generation of peptides from one protein over others. This could partly explain why some molecules were not detected. Moreover, *Peptide fingerprint* generated from a mixture of proteins could fail the algorithm of the program used to search the database.

When more than one protein is present in MALDI analysis, one may predominate over the other in the mass spectrum. This phenomenon is known as ion

suppression or signal depletion (Sterner et al, 2000). In some cases, a protein can be observed if it is loaded as a pure sample, but if it is loaded in a mixture with other proteins, its intensity may be suppressed. Ion suppression can be quite severe for complex mixtures and it could explain part of the reason for failing to identify molecules of interest.

Very faint protein bands (that are of interest) among large number of background bands may have missed excision and therefore not detected. Every effort was made to excise regions of gel that contained bands. Gel pieces were also intentionally cut and analyzed from regions at and around β1 integrin's location (130 kDa). But none of those samples yielded β1 integrin. Why MS did not detect integrin while it was detected by immunoblotting? Some recent data from our lab (Wilkins, personal communication) indicates that purified β1 integrin in solution when MS analyzed without deglycosylation, detected peptides that covered only about 50% of the molecule as opposed to 72% after deglycosylation. Peptide-masses are computed by adding the masses of aminoacids in them. Glycosylation of any residue in the peptide will offset this calculation and therefore glycosylated peptides won't be identified as that of β1 integrin by the database search program. Moreover, heavily glycosylated protein molecules may not be readily accessible to the protease enzymes. In the present experiment, none of the gel pieces were treated with deglycosylating enzymes. Thus, this factor may also have contributed to the failure of detecting integrin.

The antibody-bead induced adhesion complexes may not have contained the adhesion complexes as intact as one would have hoped for. The detergent contained in the extraction buffer may have disrupted hydrophobic interactions of the molecules in the complexes. Microscopic examination of an aliquot of beads after lysing the cell-bead clusters in the CSK buffer did not remain as aggregates but as uniform suspension. Therefore, it is reasonable to believe that the detergent disrupted the complexes leaving only a limited amount of strongly associated molecules stuck to the integrin to be detected by immunoblotting.

4.4. G3BP

One of the 25 molecules, RasGAP SH3 domain binding protein (G3BP) was of interest because of its known association with $RasGAP^{120}$. $RasGAP^{120}$ is a molecule involved in cell migration and polarization (Kulkarni et al, 2000). In addition, $RasGAP^{120}$ also regulates Ras activation, and Ras activation has been shown to stimulate membrane ruffling and lamellipodia formation (Bar-Sagi and Feramisco, 1986; Ridley et al, 1992). Ras is able to activate integrin (Zhang et al, 1996) and suppression of Ras inhibits cell spreading (Fox et al, 1994; Ridley et al, 1995; Nobes and Hall, 1999).

Ras proteins belong to a family of low molecular weight GTPases that are essential for receptor mediated signal transduction pathways controlling cell proliferation, differentiation and cytoskeletal organization (Lowy and Willumsen, 1993). They are molecular switches that in inactive state are bound to GDP and

become activated by binding to GTP (McCormick, 1994). Extracellular stimuli activate Ras through a series of protein-protein interactions involving activated receptors and adapter proteins (Pawson, 1995; Schlessinger, 1993). In the GTP-bound active state, Ras interacts with and activates downstream targets (Bourne et al, 1990; Lowy and Willumsen, 1993). Key signalling pathways downstream of Ras are the Raf/ERK kinase cascade and PI3-kinase/Akt, both being involved in proliferative and survival signal triggered by Ras (Downward J, 1998). The active or inactive conformational state of Ras is regulated by two kinds of proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Boguski and McCormick, 1993; Wittinghofer et al, 1997). GEFs increase the dissociation of GDP allowing GTP to bind to Ras. GAPs inactivate Ras by accelerating the GTP hydrolysis. Five mammalian GAPs for Ras have been described including $RasGAP^{120}$ (Trahey and McCormick, 1987; Vogel et al, 1988).

In addition to being a negative regulator of Ras, $RasGAP^{120}$ also function in the transduction cascade (Moran et al, 1990; Tocque et al, 1997). The carboxy-terminal region of $RasGAP^{120}$ has a catalytic domain, which binds Ras-GTP and accelerates GTP hydrolysis (Gawler et al, 1995; Pawson T, 1995). In the amino-terminal region, there is an SH3 (Src homology 3) domain flanked by two SH2 domains that mediate interaction with signalling proteins. A plekstrin homology (PH) domain and a stretch of amino acids involved in calcium regulated binding of phospholipids, which mediate interactions with the plasma membrane are also situated at the amino terminal (Tocque et al, 1997). $RasGAP^{120}$ induces gene

expression in a Ras-dependent manner (Medema et al, 1992; Schweighoffer at al, 1992) and is required for Ras-induced transformation (Clark et al, 1997). Cells cultured from RasGAP^{120} mutant embryos exhibited cell migration and cell polarity defects (Kulkarni et al, 2000). RasGAP^{120} has also been implicated in cytoskeletal regulation and cell survival. RasGAP^{120} knock out mouse embryos exhibit vascular defects resulting from altered cell migration (Henkemeyer et al, 1995). RasGAP^{120} *per se* is able to trigger stress fiber formation by stimulating Rho activity, and a monoclonal antibody to the SH3 domain of RasGAP^{120} blocked Rho dependent cytoskeletal reorganization and DNA synthesis induced by growth factors in mouse Swiss 3T3 fibroblasts (Leblanc et al, 1998). Microinjection of anti RasGAP^{120} antibody induced massive apoptosis in several tumor cell lines but not in normal cells (Leblanc et al, 1999). Therefore, the summary of the above data is that RasGAP^{120} is necessary for the normal behavior and survival of the cells, blocking its activity affects cell function leading to apoptosis. Upon activation, RasGAP^{120} becomes phosphorylated and associates with other proteins and phosphorylated tyrosine kinase receptors via its SH3/SH2 domains. It also complexes with G3BP using SH3 domain.

G3BP is a ubiquitously expressed 68 kDa cytosolic protein. It has 466 amino acids with sequence homology to the heterogeneous nuclear RNA-binding protein superfamily: two ribonucleoprotein motifs (RNP1 and RNP2), RG-rich domain, and acidic sequences but lack KH domain (fig. 4.1). It physically associates with the SH3 domain of RasGAP^{120}. This association occurs only in serum-fed dividing cells but not in serum-starved quiescent cells suggesting the recruitment of the complex when

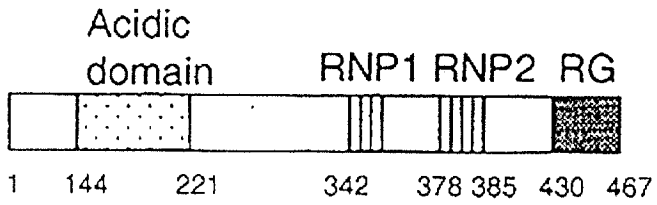

Fig.4.1: Schematic diagram of G3BP (Soncini et al, 2001)

RNP: RNA binding motif, RG: Arginine Glycine rich domain

Ras is in its active conformation (Parker et al, 1996). The recruitment of the complex and its association with the plasma membrane is consistent with a recent study showing the activation of Ras occurring at the peripheral plasma membrane (Mochizuki et al, 2001). This association is time dependent after serum addition to the cell culture, it occurs at 1 hr and 8 hr but not in between. However, the level of G3BP is constant throughout the cell cycle (Gallouzi et al, 1998). G3BP has an intrinsic endonuclease activity that was demonstrated by its ability to cleave the 3'-untranslated region of human *c-myc* mRNA (Gallouzi et al, 1998). G3BP gets extensively phosphorylated at its serine residues, and its RNase activity is dependent on this phosporylation (Gallouzi et al, 1998). Although it exhibits strong RNase activity, the primary amino acid sequence has no overall similarity to any known nucleases. Its absence of interaction with $RasGAP^{120}$ in quiescent cells corresponds to the accumulation of a soluble hyperphosphorylated form of G3BP with RNase activity in the cytosol. It is vital to rapidly degrade specific mRNAs that encode critical regulatory proteins that function only briefly during cell division. A central focus of the turnover of many cell cycle regulated mRNAs is the initiation of degradation by endonucleolytic cleavage, usually in the 3'UTR of targets. G3BP's RNase activity was demonstrated using human *c-myc* mRNA as targets and it cleaved the 3'UTR of *c-myc* and therefore initiate mRNA turnover. RNase activity in general, therefore, G3BP's RNase activity should not be considered as specific to *c-myc* mRNA but rather to attack almost any exposed U-rich motifs. In this context, G3BP acts as a growth factor (GF) sensor. When GF is withdrawn G3BP RNase is activated

and degrades mRNA whose expression is necessary for cell cycle progression. Conversely, when quiescent cells are stimulated with serum, $RasGAP^{120}$-G3BP complexes were formed and translocated to the plasma membrane. Subsequently, several mRNAs were allowed to transcribe and translate for the cell proliferation.

Even though SH3 domain of $RasGAP^{120}$ binds to G3BP, the motif of G3BP binding to $RasGAP^{120}$ is unknown. SH3 (Src homology 3) domains, first discovered in Src tyrosine kinase (Mayer et al, 1988), are widely distributed among proteins involved in the control of cell proliferation and differentiation (Mussachio et al, 1992). SH3 domain directs protein-protein interactions necessary for protein function or subcellular localization. SH3 domains contain a site composed of well-conserved aromatic residues (Booker et al, 1993; Yu et al, 1992). All SH3 domain-binding proteins contain a short contiguous proline-rich sequences that mediate binding to SH3 domains. Short proline-rich stretches of 10 amino acids were first defined as the minimal requirement for SH3 binding (Ren et al, 1993). The minimum sequences required for SH3 binding was later demonstrated as PXXP in human immunodeficiency virus *Nef* protein (Saksela et al, 1995). However, the amino acids flanking this site can provide further binding specificity (Weng et al, 1995). In G3BP there are two PXXP motifs at 294-297 (PQRP) and 431-434 (PGGP) but not proved are the region docking to SH3 domain of $RasGAP^{120}$.

All this G3BP literature is consistent with that of $RasGAP^{120}$. $RasGAP^{120}$ is required for cell survival and growth, it binds to G3BP and translocate it away from the cytosol allowing cells to divide. Therefore, one would assume that over-

expression of G3BP would halt the cell division. But it turned out to be false. Guitard et al (2001) have shown that G3BP over expression promotes cells to S phase and this transformation of cells were dependent on the presence of intact RNP domain in G3BP. They have also shown that in cells obtained from various tumours contained 5-10 times more G3BP protein than normal cells.

One recent paper describes a second binding partner for G3BP. G3BP associates with USP10 (ubiquitin specific protease 10) and inhibits its ability to disassemble ubiquitin chains (Soncini et al, 2001). Ubiquitination of protein to be degraded is fundamental for the regulation of biological pathways. The specific attachment of ubiquitin to target protein is achieved via a cascade of reactions involving ATP molecules and enzymes. Ubiquitinated proteins are recognized by the proteasome for their degradation process. Ubiquitination also acts as a targeting signal delivering the proteins to different locations in the cell and potentially modifying its activity, interactions or half life (Laney and Hochstrasser, 1999). Protein ubiquitination can be reversed (de-ubiquitination) and is catalyzed by specialized thiol proteases known as de-ubiquitinating enzymes (Ciechanover et al, 2000). More than 60 of these enzymes have been identified and they are classified in to two families; Ubiquitin C-terminal hydrolases and Ubiquitin-specific proteases (D'Andrea and Pellman, 1998).

USP deregulation has been detected in cancer. Overexpression of a mouse cDNA for USP4 leads to oncogenic transformation of NIH3T3 cells (Gupta et al, 1993). Human USP4 mRNA was found to be elevated in small cell lung carcinomas

and adenocarcinomas of the lung (Gray et al, 1995). A mouse USP over expression resulted in growth arrest indicating its growth suppressive effect (Zhu et al, 1996). The same group isolated a highly related protein that is specifically induced by IL-2 (Zhu et al, 1997). Microinjection of anti USP8 prevents fibroblasts from entering S phase in response to serum stimulation (Naviglio et al, 1998). USP10 interacts with G3BP both *in vivo* and *in vitro* and G3BP inhibits its activity (Soncini et al, 2001). But G3BP failed to interact with two other USPs tested; USP4 and USP8. This interaction was stable and appeared in both growing and quiescent human fibroblasts and also $RasGAp^{120}$ was not detected in this complex, implying this association $RasGAp^{120}$ independent. The biological role of USP10 is unknown at present. No SH3 binding site or domain was detected in USP10. The involvement of USPs in Ras signalling has been demonstrated (Taya et al, 1998; Taya et al, 1999). So the interaction between G3BP and USP10 could provide further links between ubiqitination pathway and Ras mediated signalling. The impression therefore, is that USP over expression leads to cellular proliferation and oncogenic transformation. G3BP binding to USP and inactivating its activity is consistent with G3BP's role as a negative regulator of cell proliferation.

The functional role of G3BP in adhesion complexes is still an open question. One of its binding partners $RasGAP^{120}$ is also present in the adhesion complexes. However its mere presence doesn't prove beyond doubt that it is the binding partner of G3BP in adhesion complexes. $RasGAP^{120}$-G3BP association requires the presence of serum in culture and it does not occur up to 1 hour after serum stimulation

(Gallouzi et al, 1998). However, both G3BP and RasGAP^{120} were detected at 30 minutes of adhesion complex induction irrespective of the presence or absence of serum, indicating that G3BP's recruitment in this case is RasGAP^{120} independent. There are several other proteins (Src, PI3K, Grb2, P130 and Fak) in adhesion complexes that possess SH3 domain. One of these proteins could also be the binding partner. Given the G3BP's ability to bind to non-SH3 domain containing protein USP10, one could also assume any other protein in the adhesion complex including integrin as the partner. Further work is warranted to solve this issue.

Does the presence of G3BP in the adhesion complexes make sense? Yes it does. The ability of integrin mediated cell matrix interaction to control cell survival, proliferation and differentiation have been extensively documented (Ruoslahti and Reed, 1994; Zhang et al 1995; Haack and Hynes, 2001; Oguey et al, 2000; Folkman and Moscona 1978; Menko and Boettiger, 1987; Solursh et al, 1984; Edgar et al, 1984; Dedhar, 1989). Given the major role played by G3BP in cell division, it is comprehensible that integrin mediated cellular adhesion process translocate G3BP from cytosol to the adhesion complex to allow cells to divide and differentiate.

Kulkarni et al, (2000) have shown that cells deficient of RasGAP^{120} were failed to establish complete cell polarity and migration. However, they have not demonstrated the enhanced recruitment of RasGAP^{120} to the leading edge of normal migrating cells. To complement this finding, Pazman et al, (2000) reported that r*asputin* (Drosophila homologue of G3BP) mutants displayed defects in photoreceptor recruitment and ommatidial polarity in the eye. To see if G3BP also

has some role in cell migration and polarity, a cell wound assay was performed. The result showed exclusion of G3BP in the lamellipodial structures of the migrating cell. Interestingly, G3BP was present almost up to the tip of filopodial protrusions in the migrating cells. Significance of this finding is not evident at this time. Cells that were beginning to spread on fibronectin substrate had G3BP jailed within the auspices of cortical actin, while spreading cells had less pronounced cortical actin coupled with G3BP present at the periphery. The temporal and/or spatial distribution of G3BP in spreading Vs non-spreading cells and lamellipodia Vs filopodia can be conclusively demonstrated by time-lapsed microscopy of cells transfected with GFP coupled G3BP.

Transfection of cells with G3BP lacking its RNA binding domain and/or possible SH3 binding domain could answer as to how it is recruited to the adhesion complexes. It could also determine if $RasGAP^{120}$ is its binding partner in adhesion complexes. Would these cells exhibit any defect in migration, polarity or adhesion? It would also be interesting to see how over expression of G3BP affect the above cellular behaviors. Another thing worth examining is whether G3BP is recruited only to $\beta 1$ integrin containing adhesion complexes. This can be done using beads coupled with antibodies to other integrin β subunits.

5. CONCLUSION

Even though, *understanding the cellular adhesion process* was the big picture this thesis aimed to contribute to, there were two questions that this thesis directly addressed. They were, 1) isolating the adhesion complexes intact and 2) identifying the molecules in it by mass spectrometry.

All the three approaches of adhesion complex isolation failed to provide suitable material for further analysis. However, it should be stated that, in search for a useful method, finding methods that do not work represent progress not failure. As for the identification of molecules in the adhesion complexes, mass spectrometry based identification approach was satisfactory and reliable enough to identify proteins with confidence, for instance G3BP. However, why it failed to identify some of the known focal adhesion proteins present in the complex (detected by immunoblotting) or even integrin remains mysterious. A definitive judgment about the suitability of mass spectrometry to identify proteins in a supramolecular complex could have been reached if pure adhesion complexes were available to start with. In its absence, the fairest conclusion is that a mass spectrometry based approach is satisfactory. However, the extent to which this reflects a limitation of MS or a limitation of the sample preparation methods for MS that were available, remains to be determined. However mass spectrometry has been successfully used to identify molecules in complexes such as yeast nuclear pore complexes and anaphase promoting complexes (Rout et al, 2000; Zachariae et al, 1998). Nevertheless, this work has identified G3BP as another piece of molecule in adhesion complexes. I think it is a valuable

contribution in this field, even though its role in integrin-mediated function(s) is unclear at this time. That is for now being left for the successor.

6. REFRENCES

Albeda SM, Buck CA, 1990. Integrins and other cell adhesion molecules. FASEB 4:2868-2880.

Alberts B, Bray D, Lewin J, Raff M, Roberts K, Watson JD, 1994. Molecular biology of the cell, 3rd ed. Garland publishing Inc.

Altieri DC, Edgington TS, 1998. A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/Cd18 (Mac-1). J Immunol 141:2656-2660.

Altruda F, Cervella P, Tarone G, Botta C, Balzac F, Stefanuto G, Silengo L, 1990. A human b1 integrin subunit with a unique cytoplasmic domain generated by alternative mRNA processing. Gene 95:261-266.

Andersen JS, Mann M, 2000. Functional genomics by mass spectrometry. FEBS Lett 480:25-31.

Arber S, Caroni P, 1996. Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. Genes Dev 10:289-300.

Argraves WS, Suzuki S, Arai H, Thompson K, Pierschbacher MD, Ruoslahti E, 1987. Amino acid sequence of the human fibronectin receptor. J Cell Biol 105:1183-1190.

Arroyo AG, Garcia-Pardo A, Sanches-Madrid F, 1993. A high affinity conformational state on VLA integrin heterodimers induced by an anti-b1 chain monoclonal antibody. J Biol Chem 268:9863-9868.

Avnur Z, Geiger B, 1981. Substrate-attached membranes of cultured cells isolation and characterization of ventral plasma membranes and the associated cytoskeleton. J Mol Bol 153:361-379.

Bacso Z, Bene L, Bodnar A, Matko J, Damjanovich S, 1996. A photobleaching energy transfer analysis of CD8/MHC-I and LFA-1/ICAM-1 interactions in CTL-target cell conjugates. Immunol Lett 54:151-6

Bahr U, Stahl-Zeng J, Gleitsmann E, Karas M, 1997. Delayed extraction time-of-flight MALDI mass spectrometry of proteins above 25,000 Da. J Mass Spectrom 32:1111-1116.

Balzac F, Belkin AM, Koteliansky VE, Balabanov YV, Altruda F, Silengo L, Tarone G, 1993. Expression and functional analysis of a cytoplasmic domain variant of the b1 integrin subunit. J Cell Biol 121:171-178.

Balzac F, Retta SF, Albini A, Melchiorri A, Koteliansky VE, Geuna M, Silengo L, Tarone G, 1994. Expression of β1b integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. J Cell Biol 127:557-565.

Bannikov GA, Guelstein VI, Montesano R, Tint IS, Tomatis L, Troyanovsky SM, Vasiliev JM, 1982. Cell shape and organization of cytoskeleton and surface fibronectin in non-tumorigenic and tumorigenic rat liver cultures. J Cell Sci 54:47-67

Barbadillo C, G-Arroyo A, Salas C, Mulero J, Sanchez-Madrid F, Andreu JL, 1995. Anti-integrin immunotherapy in rheumatoid arthritis: protective effect of anti-alpha 4 antibody in adjuvant arthritis. Springer Semin Immunopathol 16:427-36

Bar-Sagi and Feramisco, 1986. Induction of membrane ruffling and fluid phase pinocytosis in quiescent fibroblasts by ras proteins. Science 233:1061-1068.

Beckerle MC, 1986. Identification of a new protein localized at sites of cell-substrate adhesion. J Cell Biol 103:1679-1687.

Belkin AM, Zhidkova NI, Balzac F, Altruda F, Tomatis D, Maier A, Tarone G, Koteliansky VE, Burridge K, 1996. Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signalling potential in nonmuscle cells. J Cell Biol 132:211-216.

Bennet JS, Vilaire G, 1979. Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393-1401.

Bernstein ME, Bernstein JJ, 1977. Dendritic growth cone and filopodia formation as a mechanism of spinal cord regeneration. Exp Neurol 57:419-25

Biemann K, 1992. Mass spectrometry of peptides and proteins. Annu Rev Biochem 61:977-1010.

Blackstock W, 2000.Trends in automation and mass spectrometry for proteomics. Proteomics: a trends guide 12-17.

Boguski MS, McCormick F, 1993. Proteins regulating Ras and its relatives. Nature 366:643-654.

Booker GW, Gout I, Downing AK, Driscoll PC, Boyd J, Waterfield MD, Campbell LD, 1993. Solution structure and ligand binding site of the SH3 domain of the p85α subunit of phosphatidylinositol 3-kinase. Cell 73:813-822.

Bourne HR, Sanders DA, McCormick F, 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-132.

Brands R, Feltkamp CA, 1988. Wet cleaving of cells: a method to introduce macromolecules in to the cytoplasm. Exp Cell Res 176:309-318.

Bray D, 1992. Cell movements, Garland, New York.

Breuer D, Wagener C, 1989. Activation of phosphatidylinositol cycle in spreading cells. Exp Cell Res 182:659-663.

Brown MC, Perrotta JA, Turner CE, 1996. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. J Cell Biol 135:1109-1123.

Buehler G, 1976. Filopodia of spreading 3T3 cells. Do they have a substrate-exploring function? J Cell Biol 69:275-86

Burr, J. G., Dreyfuss, G., Penman, S., Buchanan, J., M. 1980. Association of the src gene product of Rous sarcoma virus with cytoskeletal structures of chicken embryo fibroblasts. Proc Natl Acad Sci U S A. 77:3484-3488.

Burridge K, Chrzanowska-Wodnicka M, 1996. Focal adhesions, contractility, and signalling. Annu Rev Cell Dev Biol 12:463-518.

Burridge K, Fath K, Kelly T, Nuckolls G, Turner C, 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol *4*:487-525.

Butcher EC, Picker LJ, 1996. Lymphocyte homing and homeostasis. Science 272:60-66.

Butcher EC, Scollay RG, Weissman IL, 1980. Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. Eur J Immunol 7:556-561.

Carr SA, Hemling ME, Bean MF, Roberts GD, 1991. Integration of mass spectrometry in analytical biotechnology. Anal Chem 63:2802-2824.

Castro A, Bono MR, Simon V, Rosemblatt M, 1996. Lymphocyte adhesion to endothelium derived from human lymphoid tissue. Eur J Cell Biol 70:61-68.

Cattelino A, Albertinazzi C, Bossi M, Critchley DR Curtis I, 1999. A cell free system to study regulation of focal adhesions and of the connected actin cytoskeleton. Mol Bio Cell 10:373-391.

Chait BT, Kent SB, 1992. Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. Science 257:1885-1894.

Charo IF, Nannizzi L, Phillips DR, Hsu MA, Scarborough RM, 1991. Inhibition of fibronectin binding to GPIIb-IIIa by a GPIIIa peptide. J Biol Chem 266:1415-1421.

Chace DH, 2001. Mass spectrometry in the clinical laboratory. Chem Rev 101:445-77.

Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL, 1994. Integrin mediated cell adhesion activates mitogen activated protein kinases. J Biol Chem 269:26602-26605.

Chen WT, 1981. Mechanism of retraction of the trailing edge during fibroblast movement. J Cell Biol 90:187-200

Chen WT, Wang J, Hasegawa T, Yamada SS, Yamada KM, 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J Cell Biol 103:1649-1661.

Chen YP, O'Toole TE, Ylanne J, Rosa JP, Ginsberg MH, 1994. A point mutation in the integrin beta 3 cytoplasmic domain (S752-->P) impairs bidirectional signaling through alpha IIb beta 3 (platelet glycoprotein IIb-IIIa). Blood 84:1857-65

Chernushevich IV, Loboda AV, Thomson BA, 2001. An introduction to quadrupole-time-of-flight mass spectrometry. J Mass Spectrom 36:849-65

Ciechanover A, Orian A, Schwartz AL, 2000. Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays 22:442-451.

Clark GJ, Westwick JK, Der CJ, 1997. p120 GAP modulates Ras activation of Jun kinases and transformation. J Biol Chem 272:1677-1681.

Clarke AS, Lotz MM, Mercurio AM, 1994. A novel structural variant of the human b4 integrin cDNA. Cell Adhes Commun 2:1-6.

Coller BS, 1997. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. J Clin Invest 100:S57-60

Collo G, Starr L, Quaranta V, 1993. A new isoform of the laminin receptor integrin a7b1 is developmentally regulated in skeletal muscle. J Biol Chem 268:19019-19024.

Cooper HM, Tamura RN, Quaranta V, 1991. The major laminin receptor of mouse embryonic stem cells is a novel isoform of the a6b1 integrin. J Cell Biol 115:843-850.

Cortese JD, Schwab B 3rd, Frieden C, Elson EL, 1989. Actin polymerization induces a shape change in actin-containing vesicles. Proc Natl Acad Sci U S A 86(15):5773-7

Costa M, Ochem A, Staub A, Falaschi A, 1999. Human DNA helicase VIII: a DNA and RNA helicase corresponding to the G3BP protein, an element of Ras transduction pathway. Nucleic Acids Res 27:817-821.

Critchley DR, 2000. Focal adhesions-the cytoskeletal connection. Curr Opin Cell Biol 12:133-139.

Crowe D, Chiu H, Fong S, Weissman, I, 1994. Regulation of the avidity of integrin b7 by the b7 cytoplasmic domain. J Biol Chem 269:14111-14118.

Curley GP, Blum H, Humphries MJ, 1999. Integrin antagonists. Cell Mol Life Sci 56:427-41

D'Souza SE, Ginsberg MH, Burke TA, Plow EF, 1990. The ligand-binding site of the platelet integrin receptor GPIIbIIIa is proximal to the second calcium-binding domain of it's a subunit. J Biol chem 265:3440-3446.

Damle NK, Klussman K, Aruffo A, 1992. Intercellular adhesion molecule-2, a second counter-receptor for CD11a/CD18 (leukocyte function-associated antigen-1), provides a costimulatory signal for T-cell receptor-initiated activation of human T cells. J Immunol 148:665-671

Damsky CH, Knudsen KA, Bradley D, Buck CA, Horwitz AF, 1985. Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. J Cell Biol 100:1528-1539.

Dana N, Fathallah DM, Arnaout MA, 1991. Expression of a soluble and functional form of the human b2 integrin CD11b/CD18. Proc Natl Acad Sci USA 88:3106-3110.

Dancker P, Low I, Hasselbach W, Wieland T, 1975. Interaction of actin with phalloidin: polymerization and stabilization of F-actin. Biochim Biophys Acta 400:407-14

D'Andrea A, Pellman D, 1998. Deubiquitinating enzymes: a new class of biological regulators. Crit Rev Biochem Mol Biol 33:337-352.

Dedhar S and Hannigan GE, 1996. Integrin cytoplasmic interactions and bi-directional transmembrane signaling. Curr Opin Cell Biol 8:657-669.

Dedhar S, 1989. Signal transduction via the b1 integrins is a required intermediate in interleukin-1β induction of alkaline phosphatase activity in human osteosarcoma cells. Exp Cell Res 183:207-214.

Defilippi P, Gismondi A, Santoni A, Tarone G, 1997. Signal transduction by integrins. Chapman and Hall publishing company.

Downward J, 1998. Ras signalling and apoptosis. Curr Opin Genet Dev 8:49-54.

Edgar D, Timpl R, Thoenen H, 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J 3:1463-1468.

Etzioni A, 2000. Integrins: the molecular glue of life. Hosp Pract 35:102-108.

Fässler R, Georges-Labouesse E, Hirsch E, 1996. Genetic analysis of integrin function in mice. Curr Op Cell Bio 18:641-646.

Faull RJ, Kovach NL, Harlan JM, Ginsberg MH, 1993. Affinity modulation of integrin a5b1 regulation of the functional response by soluble fibronectin, J Cell Biol 121:155-162.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM, 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64-71.

Ferrel JE, Martin GS, 1989. Tyrosine specific protein phosphorylation is regulated by glycoprotein IIbIIIa in platelets. Proc Natl Acad Sci USA 86:2234-2238.

Fischer A, Lisowska-Grospierre B, Anderson DC, Springer TA, 1988. Leukocyte adhesion deficiency: molecular basis and functional consequences. Immunodefic Rev 1:39-54

Folkman J, Moscona A, 1978. Role of cell shape in growth control. Nature 273:345-349.

Ford WL, Gowans JL, 1969. The traffic of lymphocytes. Semin Hematol 1:67-83.

Fox JE, 1994. Transmembrane signaling across the platelet integrin glycoprotein IIb-IIIa. Ann N Y Acad Sci 714:75-87

Fox PL, Sa G, Dobrowolski SF, Stacey DW, 1994. The ability of endothelial cell motility by p21ras. Oncogene 9:3519-3526.

Frelinger AL, Du XP, Plow EF, Ginsberg MH, 1991. Monoclonal antibodies to ligand-occupied conformers of integrin aIIbb3 alter receptor affinity, specificity and function. J Biol Chem 266:17106-17111.

Gailit J, Ruoslahti E, 1988. J Biol Chem 263:12927-12932.

Gallouzi I, Parker F, Chebli K, Maurier F, Labourier E, Barlat I, Caponi J, Tocque B, Tazi J, 1998. A novel phosphorylation-dependent Rnase activity of GAP-SH3 binding protein: a potential link between signal transduction and RNA stability. Mol Cell Biol 18:3956-3965.

Gao, J. X., Wilkins, J. A., Issekuttz, A. C. 1995. Migration of human polymorphonuclear leukocytes through a synovial fibroblast barrier is mediated by both beta 2 (CD11/CD18) integrins and the beta 1 (CD29) integrins VLA-5 and VLA-6. Cell. Immunol. 163:178-186.

Gates RE, Hanks SK, King LE, 1993. Focal adhesion components are enriched in ventral membranes isolated from transformed keratinocytes in culture. Biochem J 289:221-226.

Gawler DJ, Zhang LJ, Reedijik M, Tung PS, Moran MF, 1995. CaLB: a 43-amino acid calcium dependent membrane/phospholipid binding domain in p120 Ras GTPase-activating protein. Oncogene 10:817-825.

Geiger B, Bershadsky A, 2001. Assembly and mechanosensory function of focal contacts. Curr Opin Cell Biol 13:584-92

Giancotti F, Tarone G, Damsky C, Knudsen K, Comoglio PM, 1985. Cleavage of a 135K cell surface glycoprotein prevents cell adhesion to fibronectin-coated dishes. Exp Cell Res 156:182-190.

Giancotti FG, Comoglio PM, Tarone G, 1986. A 135,000 molecular weight plasma membrane glycoprotein involved in fibronectin-mediated cell adhesion. Immunofluorescence localization in normal and RSV-transformed fibroblasts. Exp Cell Res 163:47-62.

Ginsberg MH, Du X, Plow EF, 1992. Inside out integrin signaling. Curr Opin Cell Biol 4:766-771.

Golden A, Brugge JS, Shattil SJ, 1990. Role of platelet membrane glycoprotein IIbIIIa in agonist-induced tyrosine phosphorylation of platelet proteins. J Cell Biol 111:3117-3127.

Goldmann WH, 2000. Kinetic determination of focal adhesion protein formation. Biochem Biophys Res Commun 271:553-557.

Gordon SR, Staley CA, 1990. Role of the cytoskeleton during injury-induced cell migration in corneal endothelium. Cell Motil Cytoskeleton 16:47-57

Gray DA, Inazawa J, Gupta K, Wong A, Ueda R, Takahashi T, 1995. Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. Oncogene 10:2179-2183.

Gromkowski SH, Heagy W, Martz E, 1985. Blocking of CTL-mediated killing by monoclonal antibodies to LFA-1 and Lyt-2, 3. II. Evidence that trypsin pretreatment of target cells removes a non-H-2 molecule important in killing. J Immunol 134:70-7

Gromkowski SH, Heagy W, Sanchez-Madrid F, Springer TA, Martz E, 1983. Blocking of CTL-mediated killing by monoclonal antibodies to LFA-1 and LYT-2,3. I. Increased susceptibility to blocking after papain treatment of target cells. J Immunol 130:2546-51

Guitard E, Parker F, Millon R, Abecassis J, Tocque B, 2001. G3BP is overexpressed in human tumors and promotes S phase entry. Cancer Lett 162:213-221.

Gupta K, Copeland NG, Gilbert DJ, Jenkins NA, Gray DA, 1993. Unp, a mouse gene related to the tre oncogene. Oncogene 8:2307-2310.

Haack H, Hynes RO, 2001. Integrin receptors are required for cell survival and proliferation during development of the peripheral glial lineage. Dev Biol 233:38-55

Hall A, 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 10:31-54

Hakansson K, Emmett MR, Hendrickson CL, Marshall AG, 2001. High-sensitivity electron capture dissociation tandem FTICR mass spectrometry of microelectrosprayed peptides. Anal Chem Aug 73:3605-10.

Hemler ME, 1990. VLA proteins in the integrin family: structures, functions and their role on leukocytes. Ann Rev Immunol 8:365-400.

Hemler ME, 1998. Integrin associated proteins. Curr Opin Cell Biol 10:578-585.

Henkemeyer M, Rossi DJ, Holmyard DP, Puri MC, Mbamalu G, Harpal K, Shih TS, Jacks T, Pawson T, 1995. Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. Nature 377:695-701.

Hogervorst F, Kuikman I, van Kessel AG, Sonnenberg A, 1991. Molecular cloning of the human a6 integrin subunit. Alternative splicing of a6 mRNA and chromosomal localization of the a6 and b4 genes. Eur J Biochem 199:425-433.

Hogervorst F, Kuikman I, von dem Borne AE, Sonnenberg A. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118kD cytoplasmic domain. EMBO J 9:765-770.

Hogg N, Leitinger B, 2001. Shape and shift changes related to the function of leukocyte integrins LFA-1 and Mac-1. J Leukoc Biol 69:893-8

Hopkins CR, Gibson A, Shipman M, Strickland DK, Trowbridge IS, 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J Cell Biol 125:1265-74

Howe A, Aplin AE, Alahari SK, Juliano RL, 1998. Integrin signaling and cell growth control. Curr Opin Cell Biol 10:220-231.

Hughes PE, Pfaff M, 1998. Integrin affinity modulation. Trends Cell Biol 8:359-364.

Humphries MJ, 1997. Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem Soc Trans 65:79.99.

Humphries MJ, 2000. Integrin Structure. Biochem Soc Trans 28:311-339.

Hynes RO, 1992. Integrins: versatility, modulation and signaling in cell adhesion. Cell 69:11-25.

Isberg RR, Leong JM, 1990. Multiple β1-chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell 60:861-871.

Isenberg G, Small JV, Kreutzberg GW, 1979. The cytoskeleton and its influence on shape, motility and receptor segregation in neuroblastoma cells. Prog Brain Res 51:45-50

Izzard CS, Lochner LR, 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. J Cell Sci 42:81-116

Jalkanen S, Salmi M, 1993. A novel endothelial cell molecule mediating lymphocyte binding in humans. Behring Inst Mitt 92:36-43.

Jockusch BM, Bubeck P, Giehl K, Kroemker M, Moschner J, Rothkegel M, Rudiger M, Schluter K, Stanke G, Winkler J, 1995. The molecular architecture of focal adhesions. Annu Rev Cell Dev Biol 11:379-416.

Karas M, Hillenkamp F, 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60:2299-2301.

Kawasaki H, Kretsinger RH, 1995. Calcium binding proteins 1: EF hands. Protein Profile 2:297-490.

Keizer GD, Visser W, Vliem M, Figdor CG, 1988. A monoclonal antibody (NKI-L16) directed against a unique epitope on the alpha-chain of human leukocyte function- associated antigen 1 induces homotypic cell-cell interaction. J Immunol 140:1393-1400.

Kinter M, Sherman NE, 2000. Protein sequencing and identification using tandem mass spectrometry. John Wiley & sons, Inc., publication.

Kishimoto TK, Hollander N, Roberts TM, Anderson DC, Springer TA, 1987. Heterogeneous mutations in the beta subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. Cell 50:193-202

Klemke RL, Yebra M, Bayna EM, Cheresh D, 1994. Receptor tyrosine kinase signaling required for integrin avb5-directed cell motility but not adhesion on vitronectin. J Cell Biol 127:859-866.

Knezevic I, Leisner TM, Lam SC, 1996. Direct binding of the platelet integrin alphaIIbbeta3 (GPIIb-IIIa) to talin. Evidence that interaction is mediated through the cytoplasmic domains of both alphaIIb and beta3. J Biol Chem 271:16416-16421.

Kovac NL, Carlos TM, Yee E, Harlan JM, 1992. A monoclonal antibody to b1 integrin stimulates VLA-dependent adherence to leukocytes to human umbilical vein endothelial cells and matrix components. J Cell Biol. 116:499-509.

Kozma R, Ahmed S, Best A, Lim L, 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15:1942-52

Krensky AM, Robbins E, Springer TA, Burakoff SJ, 1984. LFA-1, LFA-2, and LFA-3 antigens are involved in CTL-target conjugation. J Immunol 132:2180-2

Kulkarni SV, Gish G, Geer P, Henkemeyer M, Pawson T, 2000. Role of p120 RasGAP in directed cell movement. J Cell Biol 149:457-470.

Kulkarni SV, Gish G, van der Geer P, Henkemeyer M, Pawson T, 2000. Role of p120 Ras-GAP in directed cell movement. J Cell Biol 149:457-470

Kvietys PR, Sandig M, 2001. Neutrophil diapedesis: paracellular or transcellular? News Physiol Sci 16:15-9

LaFlamme SE, Akiyama SK, Yamada KM, 1992. Regulation of fibronectin receptor distribution. J Cell Biol 117:437-447.

Laney JD, Hochstrasser M, 1999. Substrate targeting in the ubiquitin system. Cell 97:427-430.

Languino LR, Ruoslahti E, 1992. An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. J. Biol. Chem. 267:7116-7120.

Lauffenburger DA, Horwitz AF, 1996. Cell migration: a physically integrated molecular process. Cell 84:359-69

Leblanc V, Delumeau I, Tocque B, 1999. Ras-GTPase activating protein inhibition specifically induces apoptosis of tumour cells. Oncogene 18:4884-4889.

Leblanc V, Tocque B, Delumeau I, 1998. Ras-GAP controls Rho-mediated cytoskeletal reorganization through its SH3 domain. Mol Cell Biol 18:5567-5578.

Lee JO, Rieu P, Arnaout MA, Liddington R, 1995. Crystal structure of the α domain from the α subunit of integrin CR3 (CD11b/CD18). Cell 80:631-638.

Lengsfeld AM, Low I, Wieland T, Dancker P, Hasselbach W, 1974. Interaction of phalloidin with actin. Proc Natl Acad Sci U S A 71:2803-7

Lenter M, Uhlig H, Hamann A, Jeno P, Imhof B, Vestweber D, 1993. A monoclonal antibody against an activation epitope on mouse integrin chain b1 blocks adhesion of lymphocytes to the endothelial integrin a6b1. Proc Natl Acad Sci USA 90:9051-9055.

Liaw L, Skinner MP, Rains EW, Ross, Cheresh DA, Schwartz SM, Giachelli CM, 1995. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of avb3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest 95:713-724.

Lipnick RN, Iliopoulos A, Salata K, Hershey J, Melnick D, Tsokos GC, 1996. Leukocyte adhesion deficiency: report of a case and review of the literature. Clin Exp Rheumatol 14:95-8

Liu S, Calserwood DA, Ginsberg MH, 2000. Integrin cytoplasmic domain binding proteins. J Cell Sci 113:3563-3571.

Lo SH, Weisberg E, Chen LB, 1994. Tensin: a potential link between the cytoskeleton and signal transduction. Bioessays 16:817-823

Loboda AV, Krutchinsky AN, Bromirski M, Ens W, Standing KG. 2000. A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance. Rapid Commun Mass Spectrom 14:1047-1057.

Lodish, Harvey; Berk, Arnold; Zipursky, S. Lawrence; Matsudaira, Paul; Baltimore, David; Darnell, James E, 2000. Molecular Cell Biology, 4th ed. W H Freeman & Co, New York.

Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL, Ginsberg MH, 1990. A b3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249:915-918.

Lowy DR, Willumsen BM, 1993. Function and regulation of Ras. Annu Rev Biochem 62:851-891.

Mann M, Hendrickson RC, Pandey A, 2001. Analysis of proteins and proteomes by mass spectrometry. Annu Rev Biochem 70:437-73

Mayer A, Ivanov IE, Gravotta D, Adesnik M, Sabatini DD, 1996. Cell-free reconstitution of the transport of viral glycoproteins from the TGN to the basolateral plasma memebrane of MDCK cells. J Cell Science 109:1667-1676.

Mayer BJ, Hamaguchi M, Hanafusa H, 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature 332:272-275.
McCloskey JA, 1990. Methods in Enzymology, 193. Academic Press.

McCormick F, 1994. Activators and effectors of ras p21 proteins. Curr Opin Genet Dev 4:71-76.

McLafferty, 1981. Tandem mass spectrometry. Science214:280-287.

Medema RH, de Laat WL, Martin GA, McCormick F, Bos JL, 1992. GTPase-activating protein SH2-SH3 domains induce gene expression in a Ras-dependent fashion. Mol Cell Biol 12:3425-3340.

Menko AS, Boettiger D, 1987. Occupation of the extracellular matrix receptor, integrin, is a central point for myogenic differentiation. Cell 51:51-57.

Metzger WJ, 1995. Therapeutic approaches to asthma based on VLA-4 integrin and its counter receptors. Springer Semin Immunopathol 16:467-78

Michishita M, Videm V, Arnaout MA, 1993. A novel divalent cation-binding site in the A domain of the b2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell 72:857-867.

Mitchison TJ, Cramer LP, 1996. Actin-based cell motility and cell locomotion. Cell 84:371-379

Miura Y, Kikuchi A, Musha T, Kuroda S, Yaku H, Sasaki T, Takai Y, 1993. Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in Swiss 3T3 cells. J Biol Chem 268:510-5

Miyamoto S, Akiyama SK, Yamada KM, 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 267:883-885.

Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM, 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol 131:791-805

Mochizuki N, Yamashita S, Kurokawa K, Ohba Y, Nagai T, Miyawaki A, Matsuda M, 2001. Spatio-temporal images of growth factor induced activation of Ras and Rap1. Nature 411:1065-1067.

Moore MS, Mahaffey DT, Brodsky FM, Anderson RGW, 1987. Assembly of clathrin coated pits. Science 236:558-563.

Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson T, 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622-8626.

Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y, Nojima Y, 1995. Matrix/integrin interaction activates the mitogen-activated protein kinase, $p44^{erk-1}$ and $p42^{erk-2}$. J Biol Chem 270:269-273.

Mould AP, Garrat AN, Askari JA, Akiyama SK. Humphries MJ, 1995. Identification of a novel anti-integrin monoclonal antibody that recognizes a ligand-induced binding site epitope on a beta 1 subunit. FEBS Lett 363:118-122.

Mould AP, Garrat AN, Puzon-McLaughlin W, Takada Y, Humphries MJ, 1998. Regulation of integrin function: evidence that bivalent-cation-induced conformational

changes lead to the unmasking of ligand-binding sites within integrin alpha5 beta1. Biochem J 331:821-828.

Mussachio A, Gibson T, Lehto VP, Saraste M, 1992. SH3-an abundant protein domain in search of a function. FEBS Lett 307:55-61.

Nakamura S, Yakumura, H, 1989. Thrombin and collage induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets. J Biol Chem 271:8959-8965.

Naviglio S, Mattecucci C, Matoskova B, Nagase T, Nomura N, Di Fiore PP, Draetta GF, 1998. UBPY: a growth-regulated human ubiquitin isopeptidase. EMBO J 17:3241-3250.

Neubauer G, Gottschalk A, Fabrizio P, Seraphin B, Luhrmann R, Mann M, 1997. Identification of the proteins of the yeast U1 small nuclear ribonucleoprotein complex by mass spectrometry. Proc Natl Acad Sci U S A 94:385-90

Neugebauer KM, Reichardt LF, 1991. Cell surface regulation of β1-integrin activity on developing retinal neurons. Nature 350:68-71.

Ni H, Wilkins JA, 1998. Localization of a novel adhesion blocking epitope on human b1 integrin chain. Cell Adhes Commun 5:257-271.

Nobes CD, Hall A, 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81:53-62

Nobes CD, Hall A, 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol 114:1235-1244.

Oguey D, George PW, Ruegg C, 2000. Disruption of integrin-dependent adhesion and survival of endothelial cells by recombinant adenovirus expressing isolated beta integrin cytoplasmic domains. Gene Ther 7:1292-303

Otey CA, Vasquez GB, Burridge K, Erickson BW, 1993. Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. J Biol Chem 268:21193-7.

Pappin DJC, Hojrup P, Bleasby AJ, 1993. Rapid identification of proteins by peptide-mass fingerprinting. Curr Biol 3:327-332

Pardi R, Bender JR, Dettori C, Giannazza E, Engelman EG, 1989. Heterogeneous distribution and transmembrane signaling properties of lymphocyte function associated antigen (LFA-1) in human lymphocyte subsets. J Immunol 143:3157-3166.

Parker F, Maurier F, Delumeau I, Duchesne M, Faucher D, Debussche L, Dugue A, Schweighoffer F, Tocque B, 1996. A Ras-GTPase-activating protein SH3 domain-binding protein. Mol Cell Biol 16:2561-2569.

Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A, 1990. Microinjection of recombinant p21rho induces rapid changes in cell morphology. J Cell Biol 111:1001-7

Paul W, Steinwedel H, 1953. A new mass spectrometer without a magnetic field. Z. Naturforsch 8:448-450.

Pawson T, 1995. Protein modules and signaling networks. Nature 373:573-580.

Pazman C, Mayes CA, Fanto M, Haynes SR, Mlodzik M, 2000. Rasputin, the Drosophila homologue of the RasGAP SH3 binding protein, functions in ras- and Rho-mediated signaling. Development 127:1715-25

Peng J, Gygi SP, 2001. Proteomics: the move to mixtures. J Mass Spectrom 36:1083-91.

Perutelli P, Mori PG, 1992. Biochemical and molecular basis of Glanzmann's thrombasthenia. Haematologica 77:421-6

Petit V, Thiery JP, 2000. Focal adhesions: structure and dynamics. Biol Cell 92:477-94.

Plopper, G., and D. E. Ingber. 1993. Rapid induction and isolation of focal adhesion complexes. Biochem. Biophys. Res. Commun. 193:571-578.

Praeger BM, 1986. Filopodia number increases with age and quiescence in populations of normal WI-38 cells, and is correlated with drug-induced changes in proliferation in both normal and transformed populations. Mech Ageing Dev 33:221-35

Rahman N Stratton MR, 1998. The genetics of breast cancer susceptibility. Ann Rev Genet 32:95-121.

Regen CM, Horwitz AF, 1992. Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. J Cell Biol 119:1347-59

Relman D, Tuomanen E, Falkow S, Golenbock DT, Saukkonen K, Wright SD, 1990. Recognition of bacterial adhesion by an integrin: macrophage CR3 binds filamentous hemagglutinin of Bordetella pertusis. Cell 61:1375-1382.

Ren R, Mayer BJ, Cicchetti P, Baltimore D, 1993. Identification of a ten-amino acid proline-rich SH3 binding site. Science 259:1157-1161.

Retta SF, Balzac F, Ferraris P, Belkin AM, Fässler R, Humphries MJ, De Leo G, Silengo L, Tarone G, 1998. Beta1-integrin cytoplasmic subdomains involved in dominant negative function. Mol Biol Cell 9:715-731.

Ridley AJ, Comoglio PM, Hall A, 1995. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho in MDCK cells. Mol Cell Biol 15:1110-1122.

Ridley AJ, Hall A, 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389-99

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A, 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401-10

Rojas AI, Ahmed AR, 1999. Adhesion receptors in health and disease. Crit Rev Oral Biol Med 10:337-58

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas K., Zhao Y., Chait, B. T. 2000. The yeast nuclear pore complex: composition, architecture, and transport mechanism. J. Cell Biol. 148:635-51.

Ruoslahti E, Reed JC, 1994. Anchorage dependence, integrins, and apoptosis. Cell 77:477-478.

Sadler I, Crawford AW, Michelsen JW, Beckerle MC, 1992. Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. J Cell Biol 119:1573-1587.

Saksela K, Cheng G, Baltimore D, 1995. Proline-rich motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhancement growth of Nef^+ viruses but not for downregulation of CD4. EMBO J 14:484-491.

Sastry and Burridge, 2000. Focal adhesion: a nexus of intracellular signaling and cytoskeletal dynamics. Exp Cell Res 261:25-36.

Schaller MD, Parsons JT, 1994. Focal adhesion kinase and associated proteins. Curr Opin Cell Biol 6:705-10

Schaller MD, Parsons JT, 1995. p125Fak-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol 15:2635-2645.

Schlessinger J, 1993. How receptor tyrosine kinases activates Ras. Trends Biochem Sci 18:273-275.

Schmidt CE, Horwitz AF, Lauffenburger DA, Sheetz MP, 1993. Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. J Cell Biol 123:977-91

Schwartz MA, Both G, Lechene C, 1989. Effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. Proc Natl Acad Sci USA 86:4525-4529.

Schwartz MA, Schaller MD, Ginsberg MH, 1995. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 11:549-599.

Schweighoffer F, Barlat I, Chevallier-Multon MC, Tocque B, 1992. Implication of GAP in Ras-dependent transactivation of a polyoma enhancer sequence. Science 256:825-827.

Shevchenko A, Loboda A, Shevchenko A, Ens W, Standing KG, 2000. MALDI quadrupole time-of-flight mass spectrometry: a powerful tool for proteomic research. Anal Chem 72:2132-141.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. 68:850-858.

Siuzdak G, 1994. The emergence of mass spectrometry in biochemical research. Proc Natl Acad Sci USA 91:11290-11297.

Smith JW, Cheresh DA, 1990. Integrin αvβ3 ligand interaction. Identification of a heterodimeric RGD binding site on the vitronectin receptor. J Biol Chem 265:2168-2172.

Smyth SS, Joneckis CC, Paris LV, 1993. Regulation of vascular integrins. Blood 81:2827-2843.

Solursh M, Jensen KL, Zanetti NC, Lindenmayer TF, Reiter RS, 1984. Extracellular matrix mediates epithelial effects on chondrogenesis in vitro. Dev Biol 105:451-457.

Soncini C, Berdo I, Draetta G, 2001. RasGAP SH3 domain binding protein is a modulator of USP10, a novel human ubiquitin specific protease. Oncogene 20:3869-3879.

Sterner JL, Johnston MV, Nicol GR, Ridge DP, 2000. Signal suppression in electrospray ionization Fourier transform mass spectrometry of multi-component samples. J Mass Spectrom 35:385-91

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

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

Suzuki S, Naitoh Y, 1990. Amino acid sequence of a novel integrin b4 subunit and primary expression of the mRNA in epithelial cells. EMBO J 9:757-763.

Tamkun JW, DeSimone DW, Fonda D, Patel RS, Buck C, Horwitz AF, Hynes RO, 1986. Structure of Integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 46:271-282.

Tamura R, Rozzo C, Starr L, Chambers J, Reichard L, Quaranta V, 1990. Epithelial integrin a6b4 primary structure of α6 and variant forms of b4. J Cell Biol 111:1593-1604.

Tamura RN, Cooper HM, Collo G, Quaranta V, 1991. Cell type-specific integrin variants with alternative α chain cytoplasmic domains. Proc Natl Acad Sci USA 88:10183-10187.

Tarone G, Galetto G, Part M, Comoglio PM, 1982. Cell surface molecules and fibronectin mediated cell adhesion: effect of proteolytic digestion of membrane proteins. J Cell Biol 39:179-185.

Taya S, Yamamoto T, Kanai-Azuma M, Wood SA, Kaibuchi K, 1999. The deubiquitinating enzyme Fam interacts with and stabilizes beta-catenin. Genes Cells 4:757-767.

Taya S, Yamamoto T, Kano K, Kawano Y, Iwamatsu A, Tsuchiya T, Tanaka K, Kanai-Azuma M, Wood SA, Mattick JS, Kaibuchi K, 1998. The Ras target AF-6 is a substrate of the fam deubiquitinating enzyme. J Cell Biol 142:1053-1062.

Taylor JM, Richardson A, Parsons JT, 1998. Modular domains of focal adhesion-associated proteins. Curr Top Microbiol Immunol 228:135-163.

Tocque B, Delumeau I, Parker F, Maurier F, Multon MC, Schweighoffer F, 1997. Ras-GTPase activating protein: a putative effector for Ras. Cell Signalling 9:153-158.

Tomiyama Y, 2000. Glanzmann thrombasthenia: integrin alpha IIb beta 3 deficiency. Int J Hematol 72:448-54

Trahey M, McCormick F, 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science 238:542-545.

Trahey MG, Wong R, Halenbeck R, Rubinfeld B, Martin GA, Ladner M, Long CM, Crosier WJ, Watt K, Koths K, 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. Science 242:1697-1700.

Turner CE, 1994. Paxillin: a cytoskeletal target for tyrosine kinases. Bioessays 16:47-52

van der Flier A, Kuikman I, Baudoin C, van der Neut R, Sonnenberg A, 1995. A novel b1 integrin isoform produced by alternative splicing unique expression in cardiac and skeletal muscle. FEBS Lett 369: 340-344.

van Kuppevelt TH, Languino LR, Gailit JO, Suzuki S, Ruoslahti E, 1989. An alternative cytoplasmic domain of the integrin b3 subunit. Proc Natl Acad Sci USA 86:5415-5418.

Vogel US, Dixon RA, Schaber MD, Diehl RE, Marshal MS, Scolnick EM, Sigal IS, Gibbs JB, 1988. Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature 335:90-93.

Wang Q, Slegers H, Clauwaert J, 1999. Isolation of plasma membranes from rat c6 glioma cells cultivated on microcarriers. Acta Histochem 101:327-339.

Wang YL, 1985. Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J Cell Biol 101:597-602

Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S, Chapman HA, 1994. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 269:32380-32388.

Weng Z, Rickies RJ, Feng S, Richard S, Shaw AS, Schreiber SL, Brugge JS, 1995. Structure function analysis of SH3 domains: SH3 binding specificity altered by single aminoacid substitutions. Mol Cell Biol 15:5627-5634.

Wennenberg K, Lohikangas I, Gullberg D, Plaff M, Johansson S, Fässler R, 1996. B1-integrin dependent and independent polymerization of fibronectin. J Cell Biol 132:227-238.

Wiley WC, McLaren IH, 1955. Time-of-flight mass spectrometer with improved resolution. Rev. Sci. Instrum 26:1150-1157.

Wilkins JA, Li A, Ni H, Stupack DG, Shen C, 1996. Control of beta1 integrin function. Localization of stimulatory epitopes. J Biol Chem 271:3046-51

Wilkins, J. A., Li, A. Ni, H., Stupack, D. G., Shen, C. 1995. Control of b1 integrin function. J. Biol. Chem. 271:3046-3051.

Wittinghofer A, Scheffzek K, Ahmadian MR, 1997. The interaction of Ras with GTPase-activating proteins. FEBS Lett 410:63-67.

Wright J, Cooley B, Duwell J, Sieber-Blum M, 1988. Migration-related changes in the cytoskeleton of cultured neural crest cells visualized by the monoclonal antibody I-5G9. J Neurosci Res 21:148-54

Wu DY, Goldberg DJ, 1993. Regulated tyrosine phosphorylation at the tips of growth cone filopodia. J Cell Biol 123:653-64

Yamada KM, Miyamoto S, 1995. Integrin transmembrane signaling and cytoskeletal control. Curr Opin Cell Biol 7:681-689.

Yang JT, Raybum H, Hynes RO, 1993. Embryonic mesodermal defects in a5 integrin deficient mice. Development 119:1093-1105.

Yeung KK, Kiceniuk AG, Li L, 2001. Capillary electrophoresis using a surfactant-treated capillary coupled with offline matrix-assisted laser desorption ionization mass spectrometry for high efficiency and sensitivity detection of proteins, J Chromatogr 931:153-62

Yu H, Rosen MK, Shin TB, Seidel-Dugan C, Brugge JS, Schreiber SL, 1992. Solution structure of the SH3 domain of Src and identification of its ligand-binding site, Science 258:1665-1668.

Zachariae W, Shevchenko A, Andrews PD, Ciosk R, Galova M, Stark MJ, Mann M, Nasmyth K, 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science 279:1216-1219.

Zang Z, Vuori K, Wang HG, Reed JC, Ruoslahti E, 1996. Integrin activation by R-ras. Cell 85:61-69.

Zhang Z, Morla, AO, Vuori K, Bauer JS, Juliano RL, Ruoslahti E, 1993. The avb3 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin, J Cell Biol 122:235-242.

Zhang Z, Vuori K, Reed JC, Ruoslahti E, 1995. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc Natl Acad Sci U S A 92:6161-5

Zhidkova NI, Belkin AM, Mayne R, 1995. Novel isoform of b1 integrin expressed in skeletal and cardiac muscle. Biochem Biophys Res Commun 214:279-285.

Zhu X, Assoian RK, 1995. Integrin dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol Biol Cell 6:273-282.

Zhu Y, Carroll M, Papa FR, Hochstrasser M, D'Andrea AD, 1996. DUB-1, a deubiquitinating enzyme with growth-suppressing activity. Proc Natl Acad Sci U S A 93:3275-3279.

Zhu Y, Lambert K, Corless C, Copeland NG, Gilbert DJ, Jenkins NA, D'Andrea AD, 1997. DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes. J Biol Chem 272:51-57.

Ziober BL, Vu M P, Waleh N, Crawford J, Lin CS, Kramer RH, 1993. Alternative extracellular and cytoplasmic domains of the integrin a7 subunit are differentially expressed during development. J Biol Chem 268:26773-26783.