The Use of Mass Spectrometry for the Characterization of Molecules Co-purifying with Integrins

By David Puff

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

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Abstract

Cellular adhesion has been demonstrated to involve supramolecular complexes on plasma membranes of various cell types. These complexes involve not only transmembrane molecules, but rather extend into the cytoplasm to include signal transduction elements, adaptor proteins, and cytoskeletal components. Alterations in activation status or adhesion status of the cell lead to changes in specific associations within the complex. This dynamic process is believed to be mediated through post-translational events which modify the ability of various components to associate with or cross-link other elements. This reversible process of cellular adhesion is necessary for cellular migration through tissue. As adhesion molecules, integrins have a significant contribution to the functioning of these elaborate structures. Mass spectrometry has been used for the characterization of unidentified biomolecules, but this approach has not been utilized for analysis of supramolecular adhesion complexes. To test the validity of applying this technology to supramolecular complexes, proteins co-purifying with the integrin alpha-v/beta-3 were recovered from silver-stained SDS-PAGE gels and analyzed on a MALDI qQ-TOF. Using this approach, the protein(s) contained within each band on the gel could be identified. These findings support the use of mass spectrometry for the characterization of molecules co-purifying with integrins.

Introduction

Cell adhesion is a term that encompasses both cell-cell and cell-matrix interactions. These interactions involve a large number of molecules, which were first categorized on this basis, as cell adhesion molecules. Cellular adhesion events are critically involved in a variety of processes within an organism, such as fertilization and embryogenesis, development, tissue maintenance and wound repair, lymphocyte trafficking, inflammatory cell recruitment, and cancer invasion and metastasis (Rahman and Stratton, 1998). These processes and the necessary cellular interactions are mediated through different families of receptors (Albelda and Buck, 1990), including integrins, immunoglobulin superfamily members, cadherins, and selectins (Albelda and Buck, 1990). The varied effects and distribution of these molecules have implicated them in contributing to many disease processes, such as inflammatory bowel disease, leukocyte adhesion deficiency, cancer metastasis, and rheumatoid arthritis. An understanding of adhesion and the molecular interactions involved is critical for the development of more effective therapies aimed at preventing or inhibiting the progression of various disease processes.

There are a number of families of adhesion molecules, each of which is important in mediating various cellular contacts. Cell adhesion molecules of the immunoglobulin superfamily (IgSF) have important functions in cell-cell recognition in inflammation and immune responses (Springer, 1994). Cadherins are homophilic Ca^{2+}-dependent cell-cell adhesion molecules involved in specific cellular recognition and tissue morphogenesis (Takeichi, 1991). Selectins, unlike other families of adhesion molecules, are restricted to the leukocyte-vascular system, and recognize polypeptide ligand only when appropriately decorated with carbohydrate moieties (Ebnet and Vestweber, 1999). Integrins are heterodimeric adhesion molecules which

are involved in various cellular processes such as growth, differentiation, trafficking, and migration.

Evidence of intricate and dynamic molecular complexes on migrating cells is accumulating at an astonishing rate. These supramolecular complexes involve not only adhesion molecules, but rather contain cytoskeletal elements, signaling molecules, and adaptor proteins all of which contribute in some way to the overall functioning of the complex (Critchley et al, 1998). Cell adhesion is a trigger which results in molecular alterations within the complex. These changes involve post-translational modifications such as tyrosine phosphorylation, and can result in signal transduction and/or cytoskeletal assembly. Moreover, these complexes have been shown to localize to discrete microdomains of the cellular membrane termed lipid rafts. These domains, enriched in sphingolipids and cholesterol, are resistant to solubilization at low temperature by nonionic detergents and thus are often referred to as detergent-insoluble glycolipid microdomains (Simons and Ikonen, 1997). These rafts provide a specialized environment for cellular signaling, preventing inappropriate 'cross-talk' between unrelated pathways, while allowing for specific interactions to occur unimpeded. Integrins are an essential component of many of these supramolecular complexes as they initiate the process of adhesion. It is these molecules that have been chosen as the focus of this research project.

Integrins

Integrin expression appears to be universal for all nucleated cells investigated to present. That is to say that at least one integrin family member has been found on every cell or tissue studied to date (Albelda and Buck, 1990). Integrins are heterodimeric adhesion molecules comprising both an alpha and beta chain which are non-covalently linked through intermolecular disulfide bonds, and were originally

described by (Hynes, 1987). They are type-1 transmembrane molecules by definition, and there are at least 8 distinct beta integrin subunits, and 16 alpha subunits (Cary et al, 1999). These subunits combine to form at least 20 different receptors, some of which have overlapping ligand specificity (Cary et al, 1999). The combination of a particular alpha and beta polypeptide confers the ligand binding capability on the functional heterodimer (Cary et al, 1999). Electron microscopic images of purified integrins (Nermut et al, 1988) as well as chemical cross-linking experiments (Smith and Cheresh, 1988; Nermut et al, 1988) suggest an association of the amino-terminal globular domains of the alpha and beta subunits to form the extracellular ligand binding regions of the receptor. Integrins have been found to be involved in more than just ligand binding, in that they have been shown to transduce biochemical signals into the cell, and regulate a variety of cellular functions, including apoptosis, migration, and proliferation (Cary et al, 1999). This family of adhesion molecules is involved in both 'inside-out' and 'outside-in' signaling (Dedhar, 1999), being affected by or initiating various signaling pathways respectively. Since various integrin heterodimers have overlapping affinity, it was suggested that the effect of integrin ligation may indeed be determined by the protein expression of a given cell, or cell population. That is, the ligation of the same integrin in two different types of cells may result in the triggering of different biochemical pathways, and therefore have non-identical effects.

The beta subunits of all integrins are remarkably similar. The beta-G integrin isolated from sea urchin (an invertebrate) shares 37.1% identity and 57.6% similarity with human beta-1 integrin (Marsden and Burke, 1997). Certain features which are believed to impact on function are highly conserved over a wide variety of species (Marcantonio and Hynes, 1988). For example, the positions of 56 cysteine residues have been evolutionarily conserved in all of the beta subunits sequenced, with the exception of beta-4, beta-7, and beta-8 (Marsden and Burke, 1997), suggesting these

residues are critical for integrin function. The typical beta subunit possesses one membrane spanning domain, and a short cytoplasmic domain, found in the carboxy terminus of the molecule.

The alpha subunits have not been shown to exhibit conservation of sequence to the extent that the beta subunits have, but still are quite similar with respect to structure. The structure consists of a short carboxyl-terminal cytoplasmic domain, a single membrane-spanning domain, and a large globular extracellular domain, containing calcium-binding regions. Some alpha chains are composed of a large and a small polypeptide, joined by a single disulfide bond in the extracellular domain. Certain integrin alpha subunits contain an additional inserted sequence between the last calcuim binding site and the amino terminus (Hemler, 1990), which has been termed the I-domain, and has been suggested to contribute to ligand specificity.

A number of integrins have also been found to exist as different splice variants. For example, the integrin subunit beta-1 has been demonstrated to have four splice variants (beta-1A, B, C, and D), which differ only in the carboxy terminal half of the cytoplasmic domain (Belkin et al, 1996; Balzac et al, 1993; Balzac et al 1994). Splice variants are functionally significant and can determine potential associations. Such is the case for CD98 association with beta-1A, but not beta-1D. This selective splice variant association correlated with the capacity to reverse suppression of integrin activation (Zent et al, 2000). Another integrin subunit suggested to have a splice-site variation is the beta-3 molecule (Belkin et al, 1996; Balzac et al, 1993). A potential clue to the function of the splice-site variants lies within the presence or absence of the highly conserved NPXY motifs, which have been suggested to play a critical role in localization of integrins to focal adhesions (Reszka et al, 1992; Vignoud et al, 1997), almost certainly through protein-protein interactions. The identification of these alternative forms of integrin subunits will be critical for the understanding of integrin regulation.

Focal Adhesions

One example of a supramolecular adhesion complex is the focal adhesion which is a characteristic of strongly adhering fibroblasts. Fibroblasts have typically been utilized for the study of cellular migration as they have been demonstrated to form these large multi-molecular complexes which can be easily visualized using a number of microscopic techniques. Focal adhesions involve clustered integrins and associated proteins that mediate cell adhesion and signaling. Cell migration for instance is controlled by a complex set of interactions involving integrin interaction with components of the extracellular matrix as well as with cytoskeletal elements. The formation of these adhesive complexes on the plasma membrane is a result of integrin, polymerized actin filaments and associated proteins clustering together. These organized adhesive complexes serve as points of traction over which the cell can move. The mechanism by which these complexes form at the leading edge are as yet unclear. Recent findings illustrate the importance that the actin and myosin filament network and signaling molecules (such as Rho/Rac) play in the process. The mediators of tension are the integrin family of transmembrane adhesion receptors which link components of the extracellular matrix on the outside of the cell with the cell's cytoplasmic actin cytoskeleton (Regen and Horwitz, 1992). When integrin-directed adhesions are prevented, the cells round up. One group showed cellular movement over focal adhesions by studying a fluorescent beta-1 integrin chimera in living cells (Smilenov et al, 1999), confirming that these adhesions serve as points of traction. The forces responsible for this movement are generated by contraction of actin-myosin networks and can be inhibited by chemicals which prevent polymerization of cytoskeletal elements. The interaction of the actin cytoskeleton and the integrin serves as a 'molecular clutch' and thus influences the outcome of ligand interaction. The polymerization of actin at the leading edge of migrating cells is

responsible for the generation of the tension required for migration. This actin reorganization is stimulated by members of the Rho family of small guanosine triphosphatases (GTPases) (Smilenov et al, 1999). These kinases are responsible for the phosphorylation of myosin light chain kinase. The subsequent phosphorylation of myosin light chains promotes their dimerization and their interactions with actin to drive contraction. The small GTPase Rho regulates the further organization of actin into bundles and the production of the large, highly organized structures termed focal adhesions (in fibroblasts). Recent experiments illustrate the importance of Rho in these processes as inhibition of the Rho pathway with drugs that target Rho kinase results in the loss of large focal adhesions. The regulation of these complexes is critical for continued remodeling and reorganization of adhesion contacts during cellular migration as well as other processes. The role of signal transduction in these processes is illustrated by the restoration of migration to focal adhesion kinase (FAK)-deficient cells when FAK is reintroduced. However, reintroduction of a mutant form of FAK that cannot transmit signals to Src or to Cas (an activator or the Rac pathway) fails to restore cell migration (Burridge and Chrzanowska-Wodnicka, 1996). This observation suggests that the regulation of adhesion through remodeling or reorganization is a key element in the regulation of cell migration.

Cells apply force to the matrix through the integrin-cytoskeleton linkage (Choquet et al, 1997), and the integrins appear to participate in multiple cycles of binding to and release from extracellular matrix (ECM) in cell migration on specific substrata (Bretscher, 1992). Migration involves multiple steps, including extension to new regions, attachment to extracellular matrix (ECM), force generation, and release from the ECM to allow further movement and recycling (Sheetz et al, 1998). Importantly, an additional requirement for directed migration is that there must be position dependence of attachment to and release from the substratum to provide directional movement. Mechanisms for the release of integrin-mediated adhesion at

the trailing edge may be mediated enzymatically (e.g. calpain cleavage of integrin/cytoskeleton linkages) or by reversible biochemical which decreases individual integrin affinity for the cytoskeleton (Palecek et al, 1998). A phosphatase-dependent mechanism has been suggested for alpha-v/beta-3 integrin release from its ligand, vitronectin (Lawson and Maxfield, 1995). Yet another mechanism could involve loss of cytoskeletal attachment to ECM-crosslinked integrins. The unbound integrins could then diffuse away from the ECM molecules before rebinding. This mechanism relies on cytoskeletal stabilization of integrin-ECM interactions by simply maintaining the local concentration of integrins and thus allowing for temporary dissociation between integrin and ECM components (Figure I). A recent paper (Nishizaka et al, 2000) has investigated position-dependent binding and release of fibronectin-integrin-cytoskeletal interactions. It was discovered in these studies that integrins were not concentrated at the leading edge of 3T3 fibroblasts, nor did anti-integrin antibody-coated beads bind preferentially at the leading edge. However, it was observed that diffusing ligand-bound integrins attached to the cytoskeleton preferentially at the leading edge. Since integrins are not believed to bind actin directly, this may suggest the localization of various associated proteins at the leading edge allowing integrin to associate with the cytoskeleton (Figure II). Leading edge binding to fibronectin-coated beads was inhibited by cytochalasin D, suggesting that the cytoskeleton binding to integrins could alter their avidity (Nishizaka et al, 2000).

Tyrosine phosphorylation of the beta-3 integrin subunit has been shown to occur in platelets in response to integrin aggregation following stimulation of platelets. Several lines of evidence suggest that the beta-3 cytoplasmic domain tyrosine residues and/or their phosphorylation operate to mediate interactions between beta-3 integrins and cytoskeletal proteins (Jenkins et al, 1998). Although it is clear that the cytoplasmic domains of alphaIIb/beta3 are involved in signal transduction and cytoskeletal reorganization events, the precise mechanisms regulating these processes

remain to be discovered (Jenkins et al, 1998). It follows then that the phosphorylation of integrin subunits in platelets may indicate a more broadly based mechanism controlling integrin function in general. In this way, the integrin may play a direct role not only in organizing the cytoskeleton but also in transducing signals to elicit cellular responses. Integrin function has also been tied to a number of transmembrane proteins such as urokinase plasminogen activation receptor (Wei et al, 1999) and matrix metalloproteinases (Deryugina et al, 2000).

Regulation of Cellular Trafficking in the Immune System

Cellular trafficking in an immune response is also mediated through a combination of cellular adhesion molecules on leukocytes and on endothelial cells, which function in a step-wise process (Ebnet and Vestwever, 1999). A variety of adhesion molecule families, particularly the selectins, immunoglobulin superfamily molecules, and integrins, are the main contributors to the process, which is initiated by selectin dependent 'rolling' of the leukocyte along the endothelium (Ebnet and Vestwever, 1999). This rolling serves to slow down the leukocyte, allowing the cell to receive signals from the endothelial surface (Ebnet and Vestwever, 1999). The endothelium itself can upregulate expression of various adhesion molecules (e.g. ICAM-1) in response to signals from cells within the affected tissue (Sancho et al, 1999). Furthermore, it has been demonstrated that T-lymphocyte activation is inhibited by preventing LFA-1 interaction with ICAM-1 on endothelial cells (Sancho et al, 1999). During an immune response, macrophages and dendritic cells residing in local tissues produce soluble mediators, such as cytokines and chemokines, which promote cellular infiltration into the affected area (Ebnet and Vestwever, 1999). It is believed that a key role for cytokines and is to upregulate certain adhesion molecules on the surface of the endothelium in the vicinity of the inflammatory response (Ebnet

and Vestwever, 1999). Chemokines have been found to play a role in activation of the leukocytes themselves (Nishizaka et al, 2000), and they are currently the subject of intense investigation in a number of laboratories. These small molecular weight chemoattractants have also been found to initiate polarization of adhesion molecules and chemokine receptors on peripheral blood lymphocytes (Vicente-Manzanares et al, 1998). The chemokines, once secreted, can be trapped in the glycocalyx on the surface of the endothelium, thereby allowing these molecules to interact with appropriate receptors on rolling leukocytes, and mediate inside-out signaling, resulting in integrin activation (Ebnet and Vestwever, 1999). The result of this integrin activation is firm adhesion between the endothelial cell and the rolling leukocyte, mediated by LFA-1, Mac-1, and VLA-4 on leukocytes and ICAM-1 and VCAM-1 on the endothelium (Ebnet and Vestwever, 1999). The expression of both ICAM-1 and VCAM-1 on the surface of the endothelial cells are cytokine inducible. The last step of the process is termed extravasation or diapedesis, which refers to the movement of the leukocyte from the lumen of the blood vessel into the surrounding tissue. This process is poorly understood at this juncture, but it is currently being investigated. At this time, integrins are known to play a critical role in an entire range of processes, including blastula implantation, growth, differentiation, and cellular trafficking. In this respect, the study of integrins and integrin function has cross-disciplinary implications. To further our understanding of these interesting adhesion molecules, it is essential to characterize potential associations which modulate integrin function. This may provide the insight required for the development of drug therapies to treat disease processes mediated (or contributed to) by integrins, such as tumor metastasis, inflammatory bowel disease, and rheumatoid arthritis.

Integrin Signal Transduction Pathways and Cytoskeletal Associations

(An Associated Protein Overview)

An understanding of how integrins function requires a thorough inventory of molecular components involved in integrin complexes as well as the individual contributions these components make to the functioning of the complex as a whole. Integrins function in diverse processes and specific integrin heterodimers mediate adhesion to various and often multiple ligands. Each cell type has a characteristic integrin expression profile, and this is thought to reflect the individual requirements for cellular interaction. In addition, integrins expressed by resting vs. activated cells are clearly different with respect to functional activity. It is therefore necessary to shed light on the mechanisms of integrin functionality to better understand the differences and similarities in expression profiles between cell types. Integrins require specific associations with various intracellular and transmembrane molecules for functional competency. It is the goal of this project to develop approaches for the characterization of integrin associated proteins.

Integrin-dependent molecular interactions are crucial to a wide variety of processes, hence the variation with respect to ligand-specificity and cellular expression of the distinct integrin family members. However, even the relatively large diversity of integrin specificity and cellular expression alone are not believed to account for the vast multitude of potential functions that integrins mediate. Furthermore, it has been shown that the integrin cytoplasmic domains lack intrinsic kinase activity, suggesting that for integrins to be involved in signaling pathways, the association with other (signaling) molecules is absolutely required (Gumbiner, 1993). Towards this point, it has been elucidated that the effects of integrin-mediated interactions are functionally controlled by a vast number of associated proteins.

Much of the evidence supporting the presence of these integrin-associated proteins comes from in vitro binding assays, co-localization observations, and the yeast two-hybrid system. The molecules originally identified as candidates for integrin-associated proteins include a rather large number of cytoskeletal elements (Figure III), including actin (Gumbiner, 1993), vinculin (Gumbiner, 1993), alpha-actinin (Gumbiner, 1993), talin (Gumbiner, 1993), fimbrin (Gumbiner, 1993), filamin (Gumbiner, 1993), tensin (Critchley et al, 1998), and paxillin (Critchley et al, 1998). Furthermore, there is a large body of evidence which demonstrates that the cytoplasmic domains of integrins have the capacity to associate in vitro with many of these molecules (Critchley et al, 1998).

The vast majority of data accumulated on potential integrin associated proteins has been obtained from fibroblasts as they are capable of forming large molecular complexes on their cell surfaces involving integrins which are easily examined microscopically. These complexes, termed focal adhesions, involve integrin clustering and localize to areas of tight adhesion between fibroblasts and substrate (e.g. fibronectin, collagen). It is the formation of these large complexes, that allows for extensive co-localization experiments to be undertaken. Many molecules have been shown to co-localize with integrin in these focal adhesions, including both signaling and structural proteins. It is thought that the NPXY motif on the cytoplasmic domain of the integrin beta chain is responsible for integrin localization to focal adhesions and therefore may mediate these extensive interactions with cytoplasmic molecules (Reszka et al, 1992; Vignoud et al, 1997). Furthermore, ligand-independent targeting to focal adhesions is induced by deletion of the alpha subunit cytoplasmic domain (Ylanne et al, 1993), suggesting that the alpha chain cytoplasmic tail blocks cytoskeletal interaction with the beta tail. Ligand binding appears to remove this block, permitting the beta cytoplasmic tail to target to focal adhesions.

Talin is one of the first components to be recruited to nascent focal adhesions, and has been shown to bind to the cytoplasmic tail of the integrin beta-1 subunit in vitro (Knezevic et al, 1996; Horwitz et al, 1986; Tapley et al, 1989; Sharma et al, 1995) and to co-immunoprecipitate with beta-1 if GRGDS peptide is included in the lysis buffer (Vignoud et al, 1997). Talin, a cytoplasmic protein composed of ~270kDa subunits, binds to integrin beta cytoplasmic tails, vinculin, and actin filaments (Plaff et al, 1998; Knezevic et al, 1996; Sampath et al, 1998; Hemmings et al, 1996), and co-localizes with integrins at sites of substratum contact (Burridge and Chrzanowska-Wodnicka, 1996). The talin molecule consists of an N-terminal globular domain and C-terminal rod domain (Rees et al, 1990). The N-terminal domain contains an amino acid sequence similar to a region within the membrane-binding N-terminal ERM association domain in the ezrin, radixin, and moesin family of proteins (Rees et al, 1990). Binding of talin to integrin beta chain subunits (beta-1 and beta-3) is demonstrated to involve the C-terminal domain, the H-terminal head, or both (Knezevic at al, 1996), and binding is blocked by a tyrosine to alanine substitution in the first NPXY motif of the beta-1 integrin subunit (Plaff et al, 1998). Further evidence supporting a role for talin in integrin function comes from experiments performed on fibroblasts in which either anti-sense talin mRNA (Albiges-Rizo et al, 1995) or anti-talin antibodies (Nuckolls et al, 1992) were used to block the expression or function of talin revealing a strong correlation between talin and the spreading and migration.

Similar studies on vinculin, a 117kDa cytoskeletal protein, (Westmeyer et al, 1990; Rodriquez Fernandez et al, 1992) also demonstrated a strong correlation with reduction in the number and size of focal adhesions and stress fibers, again suggesting a role for this molecule in integrin functioning. In vitro studies have indicated a number of binding sites within the vinculin molecule for focal adhesion proteins, including talin (Gilmore et al, 1992), alpha-actinin (McGregor et al, 1994), paxillin

(Kroemker et al, 1994), F-actin (Huttelmaier et al, 1997), VASP (Brindle et al, 1996; Reinhard et al, 1996), tensin (Lo et al, 1994), and PKC-alpha (Hyatt et al, 1994). Embryonic Stem (ES) cells which are talin (-/-) are unable to form focal adhesions, and are subsequently unable to spread on substrate coated plates (Critchley et al,1998). This combined information on talin has been incorporated into a model for focal adhesion assembly, in which talin is envisaged to act as a key component (Critchley et al,1998). The fact that the talin dimer has the potential to cross-link integrins combined with the fact that it has actin-nucleating and cross-linking activity makes it ideally suited for such a role. In addition, talin contains 3 vinculin-binding sites. Vinculin is capable of binding to a molecule called vasodilator-stimulated phosphoprotein (VASP) (Brindle et al, 1996), which in turn can bind zyxin (Reinhard et al, 1995) and profilin, a G-actin-binding protein (Kang et al, 1997), thus providing a potential link to actin filament assembly (Critchley et al,1998).

Zyxin, another focal adhesion component also binds alpha-actinin (Crawford et al, 1992) in addition to the p85 subunit of phosphinositol 3-kinase (Shibasaki et al, 1994). Alpha-actinin, a rod-shaped, anti-parallel homodimer (100kDa subunit) binds 2 distinct regions within the beta-1 cytoplasmic domain in vitro (Otey et al, 1993), and has also been shown to co-immunoprecipitate with the beta-2 integrin subunit (Pavalko et al, 1993; Sharma et al, 1995). Murine fibroblasts, into which alpha-actinin anti-sense mRNA was micro-injected, again demonstrate a reduced ability to form focal adhesions and stress fibers (Critchley et al,1998), thereby increasing the mobility of these cells. Actin filaments themselves may link to integrins through talin, alpha-actinin, vinculin, tensin, or a combination of these (Johnson and Craig, 1995; Lo et al, 1994). These and other cytoskeletal associations with integrin subunits are believed to stabilize cellular adhesion, to regulate cell shape, morphology, and mobility, and possibly to provide a framework for signal transduction pathways (Clark and Brugge, 1995).

Additionally, integrin activation upon ligand binding (or via other pathways) results in an increase in tyrosine-phosphorylation of several different cellular proteins (Guan et al, 1991; Kornberg et al, 1991). The lack of intrinsic kinase activity of the integrin strongly implies the presence of an associated kinase (Cary et al, 1999).

One potential candidate is Focal Adhesion Kinase (FAK), which demonstrates both an increased kinase activity and tyrosine phosphorylation in response to integrin activation, which is dependent on an intact integrin beta-cytoplasmic tail (Guan et al, 1991). Furthermore, it has been demonstrated that autophosphorylated FAK serves as a binding site for Src-homology-2 (SH2) domains of the Src family members (Schaller et al, 1994) and phosphatidylinositol 3-kinase (PI 3-K) (Chen et al, 1996a). There is a rather large body of evidence supporting a functional relationship between FAK and various integrins. This association may be key in the establishment of the subsequent cascade of interaction, as the presence of the autophosphorylated tyrosine (Y397) on FAK would allow for a plethora of potential interactions to occur, including association of adaptor proteins. Adaptor proteins are small cytoplasmic proteins which contain both SH2 (phosphotyrosine binding domains) and SH3 (proline-rich binding domains). These adaptor proteins are thought to act as the link between cell surface molecules and the cytoskeletal or signal transduction elements. Adaptor proteins include ezrin, radixin, moesin, and particularly interesting new cys-his protein (PINCH) to name just a few.

PINCH has been described as an Integrin-Linked Kinase (ILK) interactive protein (Tu et al, 1999). ILK is a 59kDa molecule which demonstrates the ability to bind to and phosphorylate the cytodomain of the beta-1 integrin subunit (Hannigan et al, 1996). The binding site of the beta-1 cytodomain was originally identified using residues 738-798 as 'bait' in the yeast-2-hybrid system (Hannigan et al, 1996). *In vivo* co-immunoprecipitation indicates that ILK binds to the cytoplasmic tail (amino acid residues 788-793) of the beta-1 integrin subunit (Dedhar and Hannigan, 1996).

Furthermore, the in vitro binding of ILK with the cytodomains of other integrin subunits (beta-2 and beta-3) suggests ILK may also interact with the cytodomains of these subunits in vivo (Dedhar and Hannigan, 1996). Interestingly, the phenotype of an ILK-homologue knockout in *C. elegans* resembles that of alpha and beta subunit mutants, providing strong evidence for an important role of ILK in integrin function (Longhurst and Jenkins, 1998). ILK has been found to contain in its amino acid sequence, a plekstrin homology (PH) domain (Delcommenne et al, 1998). This domain has been implicated in the binding of phosphoinositide 3-kinase, which is a receptor-proximal intracellular effector capable of triggering signaling pathways that regulate proliferation, cell survival, protein translation, and metabolic changes (King et al, 1997). Phosphoinositide 3-kinase catalyzes phosphorylation of phosphatidylinositols to produce phosphatidylinositol 3-phosphate second messengers such as phosphatidylinositol (3,4,5) P3 (King et al, 1997).

Tetraspanins are a family of proteins termed 'molecular facilitators', which will be discussed later (Maecker et al, 1997). The name tetraspanin describes the number of times the molecules in this family span the cell membrane. Also known as TM4SF proteins, these molecules contain conserved structural motifs including putative transmembrane domains (TM1-TM4), forming one small and one large extracellular loop (EC1 and EC2), with short cytoplasmic amino and carboxyl tails (Maecker et al, 1997). This family of proteins contains at least 20 members, of which CD9, CD53, CD63, CD81, CD82, and CD151 have been found to associate with beta-1 integrins alpha-3/beta-1, alpha-4/beta-1, and alpha-6/beta-1 (1996; Berditchevski et al, 1995; Nakamura et al, 1995; Rubenstein et al, 1996; Behr and Schriever, 1995), as well as non-beta-1 integrins alpha-4/beta-7 and alpha-L/beta-2 (Hemler et al, 1996)

The term molecular facilitators is used to describe tetraspanins due to their ability mediate function through the organization of surface molecules and signaling molecules and to participate in activation, adhesion, and cellular differentiation

processes. Early evidence supporting a role for TM4SF members in cell adhesion came from the observation that anti-CD9 antibodies induced platelet aggregation (Slupsky et al, 1989). After further investigation it was discovered that the effects of CD9 on platelet aggregation involved an association with alpha-IIb/beta-3 (Slupsky et al, 1989). Other studies involving anti-tetraspanin antibodies (CD9 and CD63) or transfections have supported an involvement in adhesion and migration in a number of cell types (Shaw et al, 1995). LFA-1, VLA-3, VLA-6, and many kinases have been shown to associate with CD63 by reciprocal immunoprecipitation studies (Skubitz et al, 1996) and (Berditchevski et al, 1995). Additionally, anti-CD63 antibody-stimulated adhesion of neutrophils to endothelium is blocked by anti-LFA-1 antibodies. This data strongly suggests a role for CD63 in LFA-1 mediated adhesion. Since various tetraspanins are preferentially expressed on specific cell types, it is believed that they infer a degree of selectivity or specificity in the adhesion and migration process. For example, anti-CD81 antibodies have been shown to activate VLA-4 mediated fibronectin binding in certain B-cell lines (Behr and Schriever, 1995), and to induce LFA-1 mediated cell-cell adhesion in human thymocytes (Todd et al, 1996). Taken together, these observations indicate a functional relationship between CD81 and integrins may be cell-type specific. Further evidence for cell-type specificity is provided by the tetraspanin expression patterns on certain cells. For instance, CD37 expression is restricted to B-cells, while PETA-3 (CD151) is generally expressed on platelets, and antibodies to each of these molecules has been shown to induce integrin-mediated adhesion/aggregation in the cells mentioned (Fitter et al, 1995). Co-precipitation and co-localization experiments revealed VLA-4 association with CD53, CD63, CD81, and CD82 on a number of cell lines (Mannion et al, 1996).

From the virtually endless list of associated proteins, one can easily understand how elaborate the molecular interactions within these dynamic supramolecular complexes may prove to be. Integrins are involved in extracellular, transmembrane,

and cytoplasmic interactions and are implicated in both cytoskeletal assembly and signal transducing pathways. Activation of integrins within these networks may lead to alterations in the molecular species involved, thereby impacting on the overall functioning of the complex. It is necessary for the process of adhesion to be dynamic as various cells rely on attachment and detachment processes for diapedesis and migration through tissues. If these supramolecular complexes could not be assembled and disassembled rapidly, migration would essentially cease once a cell had adhered to a given substrate. To understand the functional characteristics of such a process, it is first necessary to take inventory of the molecules involved. Direct evidence supporting integrin associations with various components of these dynamic complexes is necessary to confirm these interactions as well as to identify previously unknown interactions and/or novel proteins. Once a molecular inventory has been taken, functional studies can then be undertaken in order to provide the functional significance for each molecular interaction. Various studies have demonstrated integrin associations either directly or indirectly. Fluorescence microscopy experiments have been utilized to investigate molecules which co-localize both on the cell membrane and cytoplasmically. Furthermore, integrin-linked kinase (ILK) has been demonstrated to co-precipitate with beta-1,2, and 3 integrin subunits (Dedhar, 1999). Additional experiments comparing ILK knockout mice to alpha/beta integrin knockout mice revealed nearly identical phenotypes, suggesting a functional relationship exists between the two.

Co-precipitation experiments have previously been utilized in combination with gel electrophoresis and a variety of detection techniques, including western blotting, coomassie-blue staining, and silver-staining, among others. These methods of detection are quite reliable and generally provide excellent repeatability. However, one limitation lies in the fact these complexes are generated in response to adhesion, thus immunoprecipitation may not be the most appropriate method to use for

examining the molecular associations involved. Another potential limitation lies in the analysis of these techniques. Following immunoprecipitation of integrin based on the alpha and/or beta chains, a gel run and detected using total protein detection methods (e.g. silver staining) may reveal major integrin bands as well as any potential associated proteins, provided these proteins were present in large enough amounts (i.e. 1-10ng). Total protein detection may therefore reveal multiple bands of unknown identity. Western blotting of a gel transferred to nitrocellulose allows the detection of specific proteins using antibodies raised against those proteins. However, the limitation here lies in the limited number of proteins that can be screened for in any given run. Although a panel of antibodies can be tested on any given run, a researcher would have to predict which molecules may be present, rather than simply analyze the results. Recent advances made in mass spectrometry has made it possible to analyze proteins/peptides extracted from gel fragments. By limiting potential cleavage of the respective proteins to specific sites allows for the synthesis of a 'peptide fingerprint', specific for any one protein.

The difficulty in undertaking such a study is the fact that distal components of the complexes may not be retained during the purification and immunoprecipitation procedures. Furthermore, proteins present in extremely low amounts within the complexes may not be detected through conventional techniques. This inevitable occurrence prevents the complete characterization of the molecular species involved through this approach alone. Under the appropriate conditions, ventral-plasma membranes of various cell clones can be obtained, which could allow for the investigation of molecular interactions in a pseudo-physiological environment. This type of an approach when used in conjunction with co-immunoprecipitation experiments could prove to be extremely useful for identifying molecular interaction. Co-localization experiments involving fluorescence microscopy can be used to indirectly confirm identified associations, while reciprocal co-immunoprecipitation

experiments can be utilized to ensure the interactions are real and the identified associations are not merely contaminants. Mass spectrometry was utilized in the approach to address issues including low protein amounts and characterization of unknown/unidentified molecules.

As there are numerous integrin heterodimers whose interactions could be investigated, this research is focussed initially on only one, the alpha-v/beta-3 integrin. This particular integrin was chosen for multiple reasons, not the least of which is the fact that the alpha-v/beta-3 integrin has been found to contribute to cellular migration through binding its ligand, vitronectin. This integrin was purified in addition to alpha-v/beta-5, which affords us the opportunity to later investigate beta chain specific associations when data from each integrin has been accumulated. Another reason for investigating alpha-v/beta-3 is that a separated and silver-stained gel containing this integrin preparation revealed multiple bands of lower molecular weight not corresponding to the published molecular weight of any known integrin subunit.

Application of Mass Spectrometry to protein characterization

Advances in mass spectrometry have allowed analysis of complex biomolecules mainly through improvements in ionization techniques(Suizdak, 1994). The difficulty had been the formation of ions in the gas phase so that they may be directed electrostatically into a mass analyzer that differentiates the ions according to their mass-to-charge ratio (m/z). Although the analyzers have not changed significantly in recent years, the advances have mainly been a product of the developments of the ESI (Electro-Spray Ionization) and MALDI (Matrix-Assisted

Laser Desorption/Ionization) ionization sources (Suizdak, 1994). Both ESI and MALDI offer a rapid and accurate means of obtaining molecular weight information on a wide range of compounds, including biomolecules. MALDI mass spectrometry, which is more resistant to the presence of salts and certain detergents and has the higher mass capability was utilized in the analysis of the purified integrin preparations. The MALDI ionization technique utilizes a pulsed UV laser beam to desorb and ionize co-crystallized sample/matrix from a metal surface. The matrix absorbs most of the incident laser energy, resulting in minimal sample damage, and the subsequent ejection of sample and matrix molecules into the gas phase (Shevchenko et al, 1996). The resultant ions are then electrostatically directed to a mass analyzer (Shevchenko et al, 1996), such as a time-of-flight mass analyzer. Analysis of both small and large molecules can be obtained using femtomole level quantities, and with careful analysis can be utilized for heterogeneous samples, making it extremely attractive for biological samples (Burlingame et al, 1994). The mass accuracy of such an approach is typically in the order of 0.1% to a high of 0.01%. Paralleling the advances in mass spectrometry has been the origin of computer programs allowing for the relatively rapid, accurate anlaysis of the obtained spectrum.

The tandem mass (ms/ms) spectral anlaysis of selected peptides from a peptide mass fingerprint indirectly allows the determination of the amino acid sequence comprising the chosen peptide. A typical analyzer arrangement for performing ms/ms uses a linear arrangement of three quadrupoles between the ion source and the detector (Figure IV). The first and third quadrupoles act as the independent mass analyzers while the second quadrupole acts as a collisional activation chamber through which ions from the first quadrupole must pass before they enter the final quadrupole. In essence, a peptide ion (termed the 'parent ion') is selected on the basis of its mass to charge (m/z) ratio and directed into the second quadrupole where collisions occur with an inert target gas (Suizdak, 1994). During this process, part of the kinetic energy of

the parent ion is converted into internal energy, which can then cause dissociation of the parent ion (Suizdak, 1994). This process generates a host of different daughter ion types depending on the charge distribution on the original parent ion (Figure V) which produces a peptide fragmentation pattern. The result is an ms/ms spectrum which is characteristic of the amino acid sequence of the peptide. Analyzing the ion masses contained within the generated fragmentation pattern provides information on the actual sequence of amino acid residues comprising the peptide. Once again, the development of software for the purpose of analyzing these fragmentation patterns generated through ms/ms has greatly improved the speed and accuracy of the results. This general approach to identifying components of complicated molecular complexes has been successfully employed to investigate other molecular associations such as the yeast nuclear core complex (Rout et al, 2000).

Proteins are commonly isolated through the use of one-dimensional or two dimensional gel electrophoresis, and visualized by staining with Coomassie Brilliant Blue or silver staining techniques. Peptides can then be produced (making use of specific proteolytic enzymes) from each band/spot on the gel that is isolated after the staining procedure. Handling the protein directly in the gel slice circumvents one step from the overall protocol and therefore avoids a sample loss for that additional step (such as transfer to PVD membrane). Following the in-gel digestion, the sequence analysis of the peptides obtained has generally been performed using Edman degradation, the goal being to accumulate sequence information and identify the protein in sequence databases or to clone the corresponding gene (Hunkapiller et al, 1984). Difficulties with this approach include chemical modification leading to the masking of neighbouring amino acids, and the potential destruction of some residues due to the harsh conditions used during the Edman degradation. The afore mentioned advances in mass spectrometry have allowed this technology to be employed for the purpose of obtaining peptide mass fingerprints to be used in database searches (Henzel

et al, 1993 and Mann et al, 1993). The peptide mass fingerprint refers to the specific array of peptides produced from a single protein digested by an enzyme with well characterized cleavage sites. Each protein that has been sequenced (and the amino acid sequence from any potential coding DNA sequence) has a unique peptide mass fingerprint (with reference to a specific enzymatic cleavage-e.g. trypsin) and that information is housed within a number of publicly available databases (e.g. NCBI). Therefore, the peptide mass fingerprint of an unknown protein can be used to search against all known or potentially encoded proteins contained within these databases. Websites such as ExPasy (www.expasy.ch) make available programs (e.g. *PeptIdent*) which allow the user to enter peptide masses obtained experimentally to search for potential matches against numerous databases. These programs also allow the user to enter experiment specific information, such as molecular weight, potential peptide modifications and error tolerance (depend on the instrument used to acquire the peptide mass fingerprint). Analysis of peptide mass fingerprint data generally yields multiple protein candidates. It is thus not uncommon for a single peptide mass to be present in any number of peptide mass fingerprints. Therefore, the programs rank the candidates according to the absolute number of peptide-mass matches between the unknown protein and a protein from the database.

Tandem mass spectrometry can then be applied to selected peptides for the purpose of confirming the protein identity originally made through the characterization of the peptide mass fingerprint. During this process, a peptide fragmentation pattern is accumulated, and is dependent on the amino acid sequence of the peptide selected. Analysis of the peptide fragmentation pattern can yield information on the actual amino acid sequence of the peptide in question. The information provided by tandem mass spectrometry can then be used in conjunction with the information provided by the peptide mass fingerprint to identify the protein(s) isolated from a given band on a gel.

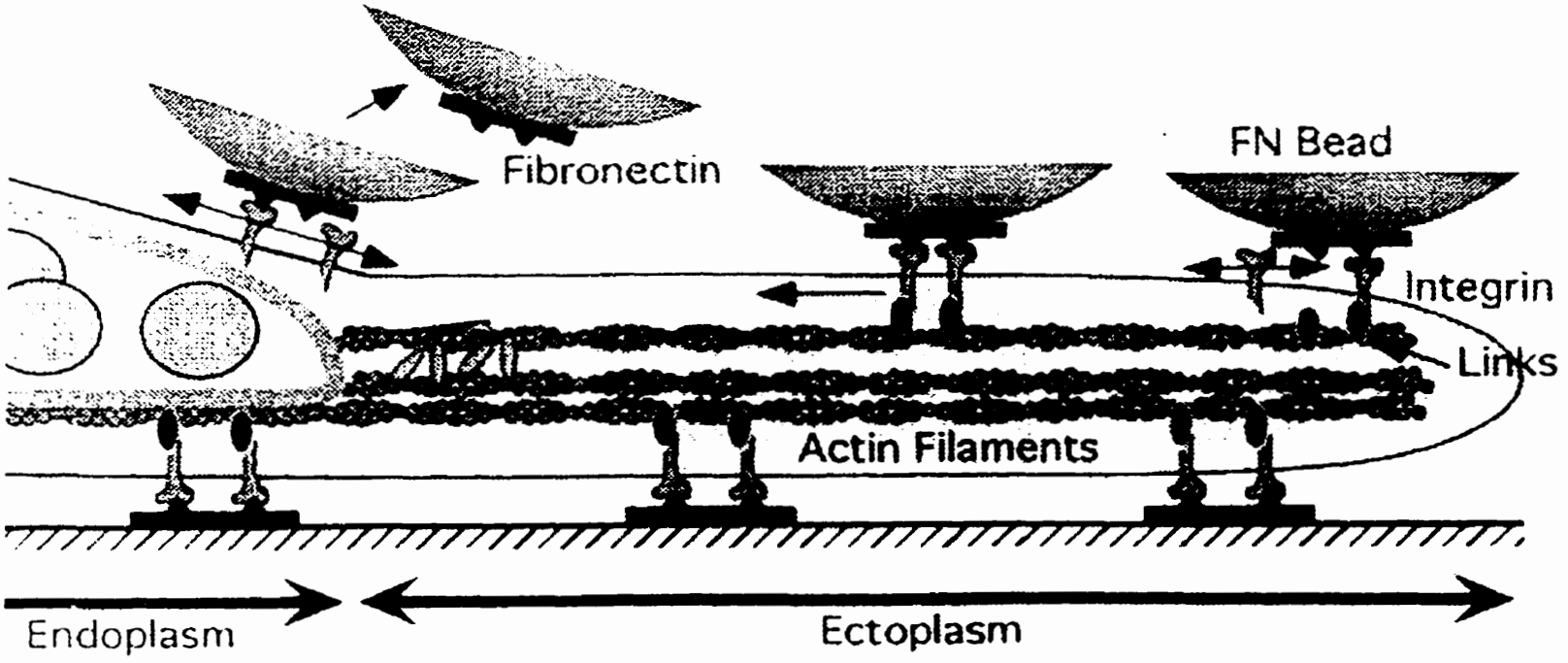


Figure I.

Schematic illustration showing that cytoskeleton binding of integrin-fibronectin complexes at the leading edge could stabilize them. A fibronectin-coated bead attaches to the dorsal surface of the leading edge and recruits a second integrin, which recruits as second link to the cytoskeleton. Because the two bound integrins are attached to the cytoskeleton, they cannot diffuse away should they release from the fibronectin. Upon release from the cytoskeleton, the integrins could diffuse away leading to fibronectin-bead release. On the ventral surface, additional components could stabilize the integrin-cytoskeleton complex perhaps in a force-dependent process. Such a position dependent binding and release cycle could aid in cell migration (Nishizaka et al, 2000).

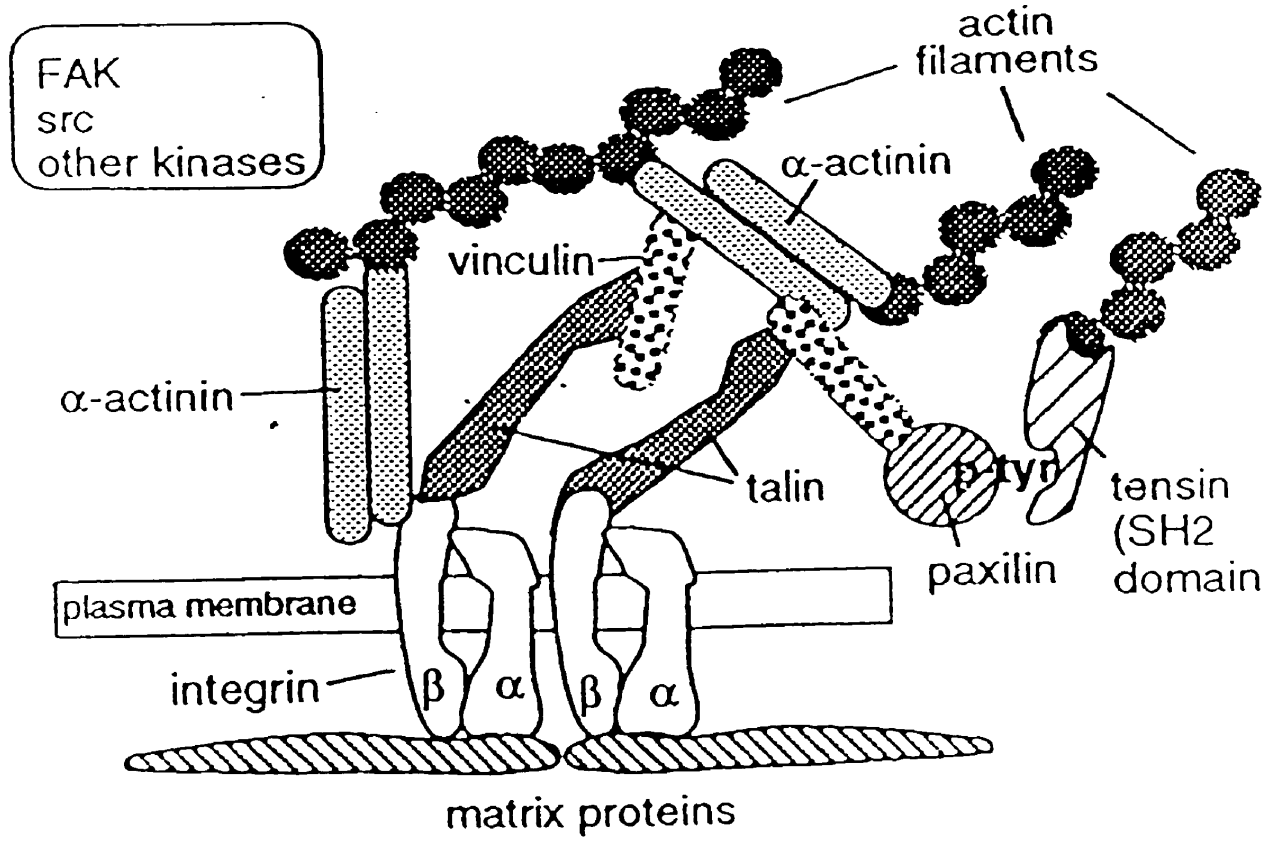


Figure II.

Connections between integrins and actin include a direct linkage via alpha-actinin and an indirect linkage via talin, vinculin, and alpha-actinin. The interaction between paxillin and tensin involves the hypothetical binding of the tensin SH2 domain to phosphorylated tyrosine residues in paxillin, which potentially provides a phosphorylation-dependent association of actin filaments with the focal adhesion proteins (Bray, 1998).

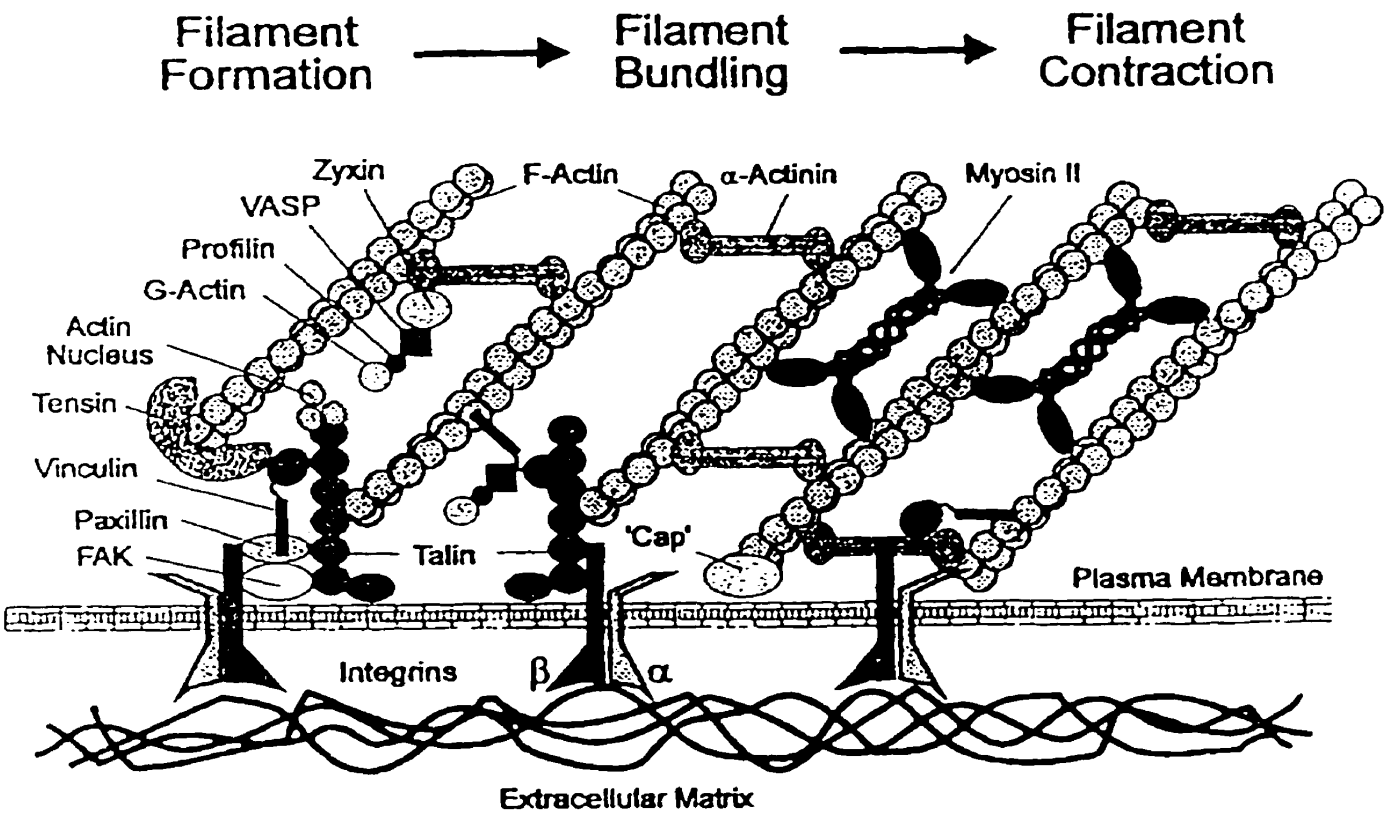


Figure III.

One interpretation of the complex interactions between integrins and their associated proteins in the context of a focal adhesion. Many proteins found within these supramolecular complexes are not thought to interact directly with integrin subunits, but rather contribute to the formation of a lattice-like protein network by bridging proteins within the complex. The binding of this large complex to the integrin occurs via the beta chain cytodomain, as illustrated (Critchley et al, 1998).

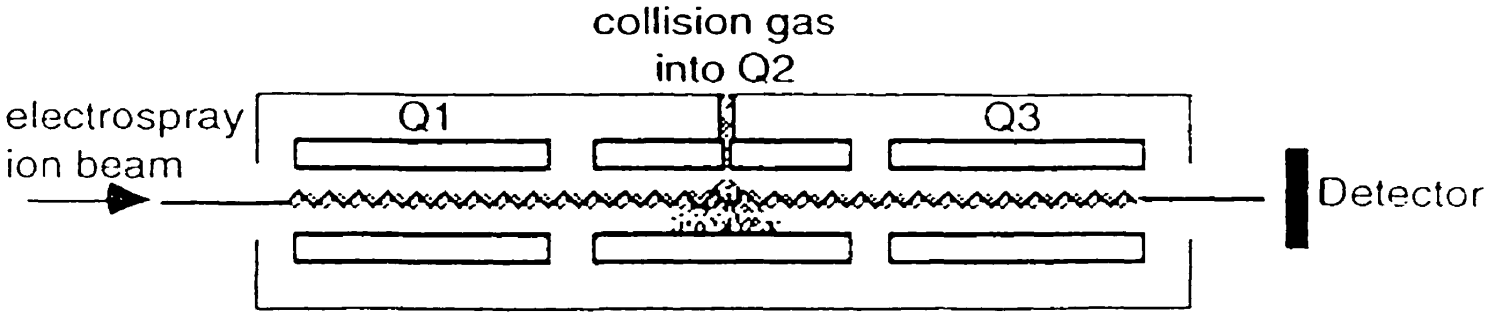


Figure IV.

A triple-quadrupole ESI mass spectrometer with ion selection and fragmentation capabilities. The first quadrupole (Q1) is used to scan across a preset m/z range or to select an ion of interest, while the second (Q2) transmits the ions while introducing a collision gas (argon) into the flight path of the ion selected by Q1. The third quadrupole (Q3) serves to analyze the fragment ions generated in the collision cell (Q2)(Suizdak et al, 1994).

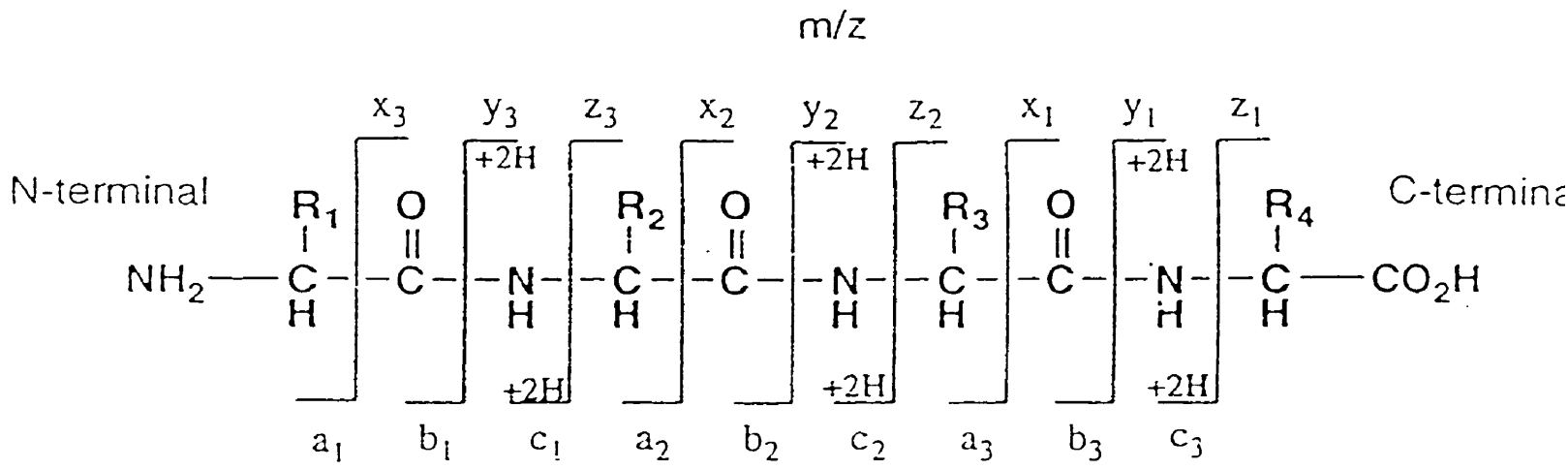


Figure V.

The fragmentation process of protonated peptides occurs through bond cleavages resulting in different classes of fragment ions. Fragment ions of type a, b, and c are generated if the charge is retained on the N-terminus of the peptide. The x, y, and z ions are formed when the charge is retained on the C-terminus (Suizdak, 1994).

METHODS AND MATERIALS

Procedure for Covalently Linking CNBr-Sepharose Beads to Antibody

Dry beads were swelled in 1mM HCl for 20min (1gram dry beads will make 3.5ml of swollen beads). The beads are then washed for 10min, again with HCl on a sintered glass filter, using gentle suction. The beads were washed at least 3 times with approximately 200ml each wash, and dried with suction on the sintered glass filter each time. The matrix was then washed with coupling buffer (containing 0.1M $NaHCO_3$, 0.5M NaCl, pH=8.3) for a few seconds, finishing as quickly as possible. The activated beads were transferred to a flask containing 60-400nM (1-5mg/ml) ligand in 30ml coupling buffer. The antibody was first dialyzed for at least 3hr against coupling buffer. The beads were incubated overnight at 4°C with the antibody (ie. Ligand). The following day, the matrix was washed with 200ml coupling buffer on a sintered glass funnel using gentle suction. The matrix was then placed in a flask and incubated for 2hr with 100ml Blocking Buffer (1M ethanolamine, adjusted to pH=8.0 with HCl) at room temperature. On a sintered glass funnel, the beads were then washed with 100ml Coupling Buffer, then with 100ml Acetate Buffer (0.1M NaOAc, 0.5M NaCl, pH=4.0). Four cycles of coupling buffer followed by Blocking Buffer were used in the final step to ensure blocking of the beads. The final wash was performed in Coupling Buffer, and the beads were stored in this Coupling Buffer (containing 0.02% NaN_3).

Integrin Purification from Placenta

Human placenta was thawed overnight at 4°C. The placenta was then washed in cold 0.9% NaCl (150 mM), the amniotic membrane dissected off, and the blood clots washed out. The placenta was then cut into small pieces and washed 4x with 0.9% NaCl. The placenta was then homogenized using commercial blender for 30 seconds at low speed followed by 30 seconds at high speed. The lysis buffer was then added, consisting of 1% NP40, 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1 mM PMSF. The lysis buffer was added at 500 ml/placenta and this mixture was then stirred at 4°C for 3 hr utilizing a simple magnetic stir bar. The lysate was then centrifuged at 3700xg for 30 minutes, and the supernatant centrifuged again at 3700xg for an additional 1 hour at 4°C. The supernatant was then collected and incubated with 5ml covalently-linked anti-integrin antibody-beads per liter of supernatant at 4°C overnight. (The supernatant could be changed by letting the beads settle down for 1 hr).

The beads were washed 5-6 times (20:1 / wash buffer:bead volume) with buffer containing 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1% NP40. Following the extensive washing of the beads (3-4x), the integrins were eluted with buffer containing 100 mM glycine-Hcl (pH 2), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM n-Octyl β-D-Galactopyranoside (Sigma). The elute was collected into 2M Tris-HCl (pH 8.2) at 2.5 ml Elute/0.5 ml Tris-HCl, mixed and a sample taken for gel electrophoresis. The remainder of the elute was stored at -20°C.

Pooled samples of integrins were dialysed against 150 mM NaCl, 25mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM mM n-Octyl β-D-Galactopyranoside at 4°C. The dialysis buffer was changed several times. Protein and functional assays were performed to ensure quantity and quality of the purified integrin. Integrin purification was performed by Keding Chen.

In-Gel Digestion of Proteins for MALDI-TOF

Bands appearing following the Modified Silver Stain Procedure(Bio-Rad) were trimmed from the gel using a scalpel, and each placed into separate microfuge tubes containing 5% acetic acid. For all steps involving handling of the gel, fresh powderless gloves were used in order to prevent/reduce sources of possible contamination (such as human keratin). A clean scalpel blade was used for excising bands of interest, and the blade(s) were washed between bands in an attempt to prevent cross-contamination. The bands were cut into fairly small fragments in order to maximize the surface to volume ratio. Following the transfer of the gel fragments to mircofuge tubes, the fragments were washed once by vortexing briefly in 50μL of 0.1M ammonium bicarbonate. The tubes were then centrifuged for 5-10seconds at 500rpm. The liquid was removed and discarded. The gel fragments were subsequently shrunk in 3-4 gel volumes (~15μL for average mini-gel bands, and ~40μL for standard gel sizes) of acetonitrile (Sigma) for 15minutes at room temperature. The fragments were centrifuged for 5 seconds and the liquid removed/discarded. Drying of the fragments was performed in a Speedvac for about 5minutes without heat. The fragments were swelled in 2-3 gel volumes of 0.1M ammonium bicarbonate containing 10mM DTT for 1 hour at 56°C in order to reduce cysteine residues. The tubes were briefly centrifuged, and the liquid discarded after allowing the tube to cool to RT. Two to three gel volumes of 0.1M ammonium bicarbonate containing 55mM iodoacetamide (Aldrich Chemicals) were added to alkylate free cysteines and the tubes then incubated at RT, wrapped in tin foil for 45min. with occasional vortexing. The samples were briefly centrifuged and the liquid discarded. The gel fragments were washed by vortexing briefly in 100μL of 0.1M ammonium bicarbonate. The washing solution was removed and the gel fragments shrunk in acetonitrile. The gel pieces were swollen in 2-3 volumes of digestion buffer (50mM ammonium bicarbonate, 5mM $CaCl_2$, 5ng/uL excision grade trypsin (Calbiochem)) on ice for 45min, and more solution was added after 20min. if the gel fragments appeared to be dry. As much of the digestion buffer as possible was removed and replaced with 5-10μL

of digestion buffer without trypsin. The samples were allowed to incubate at 37°C overnight. The tubes were then centrifuged briefly and the supernatants transferred to clean tubes. The peptides were then extracted by soaking the gel fragments in 2-3 gel volumes of 20mM ammonium bicarbonate for 20min. at RT, with occasional vortexing. The samples were centrifuged and combined the extracts combined with the supernatants from the overnight digestion (in the previous step). The peptide fragments were then further extracted by treating with 2-3 gel volumes of 5% formic acid (Sigma) in 50% acetonitrile for 20min. at RT. This extraction was repeated once more, then all the extractions were combined. The combined extracts (from both NH_4CO_3 and formic acid/acetonitrile) were then dried down in a vacuum centrifuge (without heat) and stored at -20°C until submitted for Zip-Tip (Millipore) desalting and mass spectral analysis.

Final Sample Preparation and Instrumentation

Lyophilized samples were reconstituted in 10uL of 20mM ammonium bicarbonate, then drawn up and expelled 10x across the C18 column contained within ZIP-TIPs to maximize protein binding. The ZIP-TIPs were subsequently washed with ammonium bicarbonate buffer (20mM) and eluted with 2.0uL of 5% formic acid+50% acetonitrile. In an attempt to prevent formlyation of the peptides, 0.1% trifluoroacetic acid (TFA)+50% acetonitrile was used for the elution buffer. TFA was then added (if not used during elution) to 0.1% to promote ionization of the proteins from the matrix and facilitate acquisition of mass spectra.

Incorporation of the peptide samples into a solid matrix was performed immediately prior to acquisition of the mass spectrum from the sample. A total of 0.6uL of dihydroxybenzoic acid (DHB) was placed on the probe tip and allowed to crystallize at room temperature. Following crystallization, 0.6uL of the purified protein (extracted from individual bands of the gel) was place directly on top of the solidified crystal matrix. The solvent was allowed to evaporate prior to obtaining the mass spectrum of the peptides. Mass spectral analysis of isolated peptides was conducted using MALDI quadrupole/TOF (QqTOF) tandem mass spectrometer, adapted from a prototype of the

Sciex QStar. Up to 40 MALDI samples can be deposited onto a custom-built, computer-controlled 2D positioner. Ions are produced with a UV N_2-laser operating at 20 Hz, and focused to a spot-size of about 0.3 mm^2. The acceleration voltage of the TOF section has been increased to 10 kV from 4 kV to improve the detection efficiency for singly-charged MALDI-generated ions.

Analysis of Mass Spectra

The mass fingerprints were analyzed using freely available internet software programs. *PeptIdent* (www.expasy.ch) and *ProFound* (www.prowl.com) were the two programs used to analyze the acquired peptide mass fingerprints. Through the use of two separate programs for the analyses, our confidence in the obtained results increased. Specific peptide masses were then individually selected from the peptide mass fingerprints for tandem mass spectrometry in order to gain sequence information on the selected peptide. For analysis of peptide fragmentation patterns acquired through the use of tandem mass spectrometry, two independent programs were again used to increase confidence in the obtained results. *PepFrag* (www.prowl.com) and *MsTag* (prospector.ucsf.edu) were used to confirm the results obtained the other. *FindMod* (www.expasy.ch) was utilized for the purpose of identifying various chemical modifications to unidentified peptides that may have adversely affected their identification through the use of the afore mentioned programs.

Results

Preliminary Studies:

As a preliminary test of the protocol, a sample of purified bovine serum albumin (BSA) was run on an SDS-PAGE gel. The gel was silver-stained and a band at approximately 66kDa was cut from the gel and processed for analysis by mass spectrometry. Figure 1 represents a photograph of a silver-stained10% SDS-PAGE gel used to separate components of purified bovine serum albumin (BSA). Ten micrograms (151.5pmol) of BSA were loaded onto the gel and the dominant band selected for analysis via mass spectrometry. The major band was selected on the basis of molecular weight similarity to the theoretical value for BSA, as well as band intensity. Figure 2 shows the peptide mass fingerprint of protein(s) extracted from the selected band. The pattern of peaks represented in this fingerprint were converted simply to peptide masses and analysis by computer programs *PeptIdent* and *Profound.* The analysis led to the conclusion that the peptide masses present in the fingerprint match the digestion pattern that would be generated through the tryptic digest of bovine serum albumin. Figure 3 displays the results obtained from the bioinformatic analysis using the afore mentioned *PeptIdent.* The same results were obtained when the analysis was performed on the same set of peptide masses using *Profound.* The analysis of the peptide mass fingerprint via two independent programs identified the protein as bovine serum albumin. In fact, 12 peptides were identified as belonging to purified bovine serum albumin, and these peptides represent just over 25% of the molecule. There is a great deal of experimental information which must be entered and parameters set for efficient use of the fingerprint analysis program(s). For interpretation purposes, Figure 3 has been labelled with small numbers and the corresponding input parameter described here:

(1) Name given to sample

(2) Database and species against which the peptide mass fingerprint was searched

(3) pI and Molecular weight of the protein (will be bracketed by 30%)

(4) List of experimentally acquired masses from peptide fingerprint

(5) Maximum allowable difference between experimental peptide mass and peptide mass from database

(6) Minimum number of matching peptides required to show respective candidate

(7) Maximum number of matching protein candidates to show

(8) Monoisotopic peaks were labelled and MALDI generates peptides with a single positive charge

(9) Enzyme used for protein digestion and number of cleavage sites the enzyme was allowed to miss within a single peptide

(10) Known or suspected chemical modifications to peptide(s) due to sample preparation (treatment with iodoacetamide and exposure to acrylamide monomers)

(11) Allowance for oxidized methionines within peptides

(12) Score represents the certainty with which the program has identified the correct protein (based on number of matching peptides and protein coverage). Best possible score is 1.00.

(13) Total number of peptide masses from the peptide fingerprint that match the respective protein (from database)

(14) SwissProt accession number of matching protein (from database)

(15) SwissProt name assigned to matching protein from database

(16) Description of matching protein from database

(17) pI of matching protein from database

(18) Molecular weight of matching protein from database

(19) User mass column represents the experimentally acquired masses used in search against database

(20) Mass of matching peptide from candidate molecule in database

(21) Difference in parts per million (ppm) between the experimental mass and matching mass from candidate protein

(22) Number of missed cleavages within a matching peptide

(23) Modification(s) to peptide allowing for respective masses to match

(24) Position of peptide within candidate protein from database

(25) Amino acid sequence of matching peptide from candidate molecule

(26) Difference in molecular weight between user entered mass and theoretical mass of candidate molecule

(27) Percentage of candidate protein covered by matching peptides

(28) Complete amino acid sequence of candidate molecule. Indicated in capital letters are the amino acids accounted for with matching peptides

This preliminary test demonstrated successful identification of approximately 150picomoles of bovine serum albumin. This preliminary data suggests that the technique itself works and that the current protocol can be applied to other samples for protein characterization.

Results from Alpha-v/Beta-3 Integrin Preparations:

The integrin alpha-v/beta-3 was purified from human placenta as previously described. The purified integrin sample was run a number of times on SDS-PAGE gels and silver stained. In order to obtain the best separation of proteins through a broad mass range, gel concentration and voltage were delicately adjusted . The gradient gel shown in Figure 4 (left) represents the conditions which maximized

separation and minimized diffusion of proteins in the preparation under reducing conditions. Figure 4 (right) shows the same gel following band excision. The presence of various bands at molecular weights uncharacteristic of any integrin subunit was surprising. The alpha-v/beta-3 integrin was purified under quite stringent conditions not thought to favour any type of molecular association. These various lower molecular weight bands were thought to possibly represent associated proteins which interact strongly with the purified integrin. The bands which could be visualized were cut from the gel and processed for mass spectral analysis. Based on molecular weight and intensity, band A was believed to contain the integrin alpha-v subunit. Band B was believed to contain the integrin beta-3 subunit, again based on molecular weight and intensity. Each gel fragment was analyzed by mass spectrometry to generate the respective peptide mass fingerprints. The proteins contained within the bands were identified by their fingerprints. Figure 5 shows the peptide mass fingerprint of band A (see Figure 4), a protein later identified as the 130kDa alpha-v integrin subunit. The height of the peak represents relative abundance of the peptide ions observed, while the x-axis represents the mass to charge (m/z) ratio. For MALDI, the charge obtained by an individual peptide is almost always +1, therefore the m/z can simply be interpreted as the mass of the corresponding peptide. Individual peptide peaks were labelled based on peak intensity using a program called *TOFMA* (Time Of Flight Mass Analysis). Only the monoisotopic peaks from an individual peptide mass 'cluster' were labelled. This allows consistent interpretation of mass spectra. The mass values of these peaks were then used to search the database(s). Virtually every peptide from the fingerprint could be accounted for as belonging to the integrin alpha-v subunit. The few peptides which did not correspond to the alpha-v integrin subunit were background peaks also appearing in the fingerprint of the control gel piece (data not shown). The analysis of the peptide mass fingerprint was performed using *PeptIdent* and *Profound*. Utilizing two independent programs,

which search against different databases, allows for a more confident analysis. The analysis is accepted with greater confidence when equivalent results are obtained using independent programs which search distinct databases. Identical results were obtained from each program and are shown in Figure 6 and 7 respectively.

Following the identification of the fingerprint as belonging to the alpha-v integrin subunit, tandem mass spectrometry was carried out on a select number of peptides identified as belonging to the alpha-v integrin subunit. Figure 8 shows the fragmentation pattern of the 1413.8Da peptide selected from the peptide fingerprint. To confirm the origin of the peptide, the analysis of the fragmentation pattern should yield the same sequence originally suggested by *MsTag/PepFrag* analysis of the peptide fingerprint. Monoisotopic peaks were labelled manually using *TOFMA* on the basis of intensity. *TOFMA* is a program developed by Werner Ens (Department of Physics, University of Manitoba), allowing visualization of acquired mass spectra as well as various spectral manipulations, including peak labelling. Comparison of the generated fragmentation pattern with databases containing all known peptide sequences was performed using *MsTag* and *PepFrag*. The the analysis of tandem mass spectra for selected peptides confirmed the presence of the alpha-v integrin subunit the band A. *MsTag* results obtained from the analysis are shown in Figure 9. Important features of MsTag output files are indicated by numbers in parentheses. Many categories are self explanatory or have been described already for *PeptIdent*. A brief description of critical variables is given here for interpretation purposes:

(1) The number of proteins from the database whose mass was within the range entered for the unknown protein.

(2) The mass of the parent peptide selected for tandem mass spectrometry including the allowable mass difference for searching against the database.

(3) Masses of fragmentation ions generated through tandem mass spectrometry of the selected parent peptide.

(4) Types of ions considered by *MsTag* when searching for matching peptide sequence from database.

(5) The expected chemical status of the N-terminus of the peptide.

(6) The expected chemical status of the C-terminus of the peptide.

(7) Total number of ions from the experimental fragmentation pattern not matching the suggested amino acid sequence.

(8) Suggested sequence for the selected peptide (based on fragmentation pattern analysis).

(9) Monoisotopic mass of the suggested amino acid sequence.

(10) Mass difference between the user entered peptide mass and the calculated mass of the suggested amino acid sequence.

(11) Number assigned to the alpha-v integrin subunit in the MS-Digest Index.

(12) Characterization of the individual fragmentation ions with respect to ion series or internal ion sequence.

Band B (see Figure 4) was suspected to contain the beta-3 subunit as it appeared at approximately 120kDa, the expected molecular weight range for this protein subunit. This band was analyzed successfully utilizing the same procedure as described above. Again, *TOFMA* was used in tandem with the mass spectrometer for peptide fingerprint creation (Figure 10). The analysis was then performed using *PeptIdent* and *Profound* (Figure 11 and 12 respectively) and the protein was identified as the beta-3 integrin subunit. Several peptides masses were again selected for fragmentation by tandem mass spectrometry, an example of which is the 1820.8Da peptide (Figure 13). The subsequent analysis of the fragmentation pattern was performed utilizing *MsTag* and *PepFrag* (Figures 14 and 15 respectively) as previously described. The results from tandem mass spectrometry were consistent with the analysis of the peptide fingerprint for the identified beta-3.

Figure 16 is the *PeptIdent* output file generated when all peptides identified as alpha-v are incorporated into a single analysis. From fingerprint analysis, a total of 40 peptides could be identified as belonging to the alpha-v subunit. In a number of cases, more than one peptide was shown to cover a particular amino acid sequence. Chemical modification of some peptides resulted in the presence of multiple peaks corresponding to an individual peptide. Alternatively, missed cleavages can lead to overlapping protein sequence coverage by individual peptides. Taking each of these possibilities into consideration, the 56 peptide peaks were identified as integrin alpha-v and found to cover 43.1% (accounting for 463/1018 amino acids) of the entire protein sequence. This calculation does not take into account the 30 amino acid signal sequence as this portion would not be present in the mature protein. In addition, tandem mass spectral analysis was performed on 14 of these 56 peptides, each fragmentation pattern confirming the amino acid sequence suggested by analysis of the fingerprint. It should be noted however that the most peptides recovered from the alpha-v band in a single experiment was 22. This corresponded to a 27.4% coverage of the entire protein sequence.

Similar analysis was performed on the integrin beta-3 fingerprint. In this instance, a total of 34 peptides covering 38.7% (accounting for 298/762 amino acids) of the molecule were identified (Figure 17).

A single alpha-v/beta-3 preparation was run four times and results from analysis of the mass spectra were consistent between runs. Another preparation of alpha-v/beta-3 was analyzed in exactly the same way as the first preparation, again confirming the identification of alpha-v and beta-3 from major bands.

The lower molecular weight bands of lesser intensity (see Figure 4) were not found to correlate well with any protein in SwissProt or NCBI-nr databases based on fingerprint analysis. Figure 18 represents the peptide mass fingerprint from band G of the gel. This particular fingerprint is used as a representative example for the

remaining fingerprints obtained from bands on the gel (see Figure 4). Each band did indeed have a unique fingerprint, however the analysis of peptides from each individual band gave similar results to those from band G. The fingerprint of band G was analyzed through selection of individual peptides for MS/MS and fragmentation pattern analysis. Tandem mass spectrometry of several peptides contained within these bands identified many as originating from either the alpha-v or beta-3 integrin subunits. The respective peaks of the fingerprint are labelled accordingly as alpha-v, beta-3, T (trypsin), or B (background). Identification of a few of these peptides was fairly straight forward, but the vast majority of peptides found within these fingerprints were more difficult to identify. A more thorough analysis of the spectrum in the context of potential chemical modifications to peptides during processing was required. Table 1 summarizes the characterization of peptides recovered from each band of the gel. Again, Table 1 is used here to represent the typical results obtained from any single experiment in a series of identical alpha-v/beta-3 sample runs.

During the analysis of the peptide fingerprints generated from in-gel digestion of individual bands, it became apparent that peptides belonging to the integrin alpha-v subunit were present in multiple bands. Tandem mass spectrometry performed on selected peptides and results from the analysis of the fragmentation patterns generated pointed to the presence of the integrin alpha-v subunit. Since the molecular weight of many of these bands was well outside the predicted molecular weight of the alpha-v band, it was suggested that the alpha-v subunit may have undergone limited proteolysis during the immunopurification procedure, thus leading to the formation of alpha-v fragments. To visualize the extent to which integrin alpha-v breakdown components appeared, the alpha-v/beta-3 preparation was run on a gel and transferred to nitrocellulose. The membrane was then detected using a panel of anti-alpha-v antibodies, thus allowing detection of the maximal number of breakdown fragments

(data not shown). The detected membrane illustrates the presence of alpha-v integrin subunit components in multiple bands along the length of the gel.

Various peptides from the lower molecular weight bands were selected for sequence analysis via tandem mass spectrometry. While the vast majority of peptides extracted from these bands were found to correspond to either alpha-v or beta-3 integrin subunits, one 'associated protein' was identified. This protein was identified through the fragmentation patterns of two individual peptides (1790.9 and 1954.1) as actin. Figure 19 displays the *PepFrag* analysis of the 1954.1 kDa peptide which was identified as an actin peptide. Unfortunately, no other peptides could be identified which corresponded to actin or any other potentially associated protein.

Potential chemical modifications were taken into account based on the series of chemical exposures during sample preparation. Chemical modifications found to be important during this investigation are shown in Figure 20 and calculated using the program *FindMod* (data not shown). An example of tandem mass spectrometry analysis of a selected peptide suspected to have undergone chemical modification is shown in Figure 21. Figure 21 represents the analysis of a 2381.2 Da peptide using *MsTag*. This particular peptide was found to be chemically modified by formic acid. This formic acid modification resulted in a mass shift of 28 Da between the selected peptide and unmodified parent peptide (2353.2 Da). Deamination was also identified as a relevant chemical modification in the analysis of peptide fingerprints obtained using our current protocol. This modification results in a loss of an NH_3-group (17Da) from the parent peptide, the *PepFrag* analysis of intact peptide is shown in Figure 22a, while the peptide having lost the NH3-group is identified in Figure 22b. Chemically modified peptides were commonly found in peptide fingerprints. In actual fact, 56 peptides were recovered from gel fragments which could be identified as alpha-v. However, after taking into consideration all of the chemical modifications, only 40 of

these corresponded to unique peptides. Table 2 illustrates the relative numbers of peptides with specific chemical modifications that were identified as alpha-v.

Further experiments were conducted to determine the effect of gel piece maceration (prior to peptide extraction) on peptide recovery from the gel pieces. Alpha-v alone was investigated with respect to this technical alteration. It was found that gel maceration immediately prior to peptide extraction increased the total number of peptides identified as alpha-v that were recovered from the respective gel fragments. The maceration experiment was performed a single time and compared to the alpha-v peptide recoveries from 4 experiments run under the same conditions, without gel maceration. Table 3 represents this comparison. It should be noted that while this comparison is based on a single experiment involving gel maceration, peptide recovery from this one experiment surpassed the recovery efficiencies of all four non-macerated gel extractions.

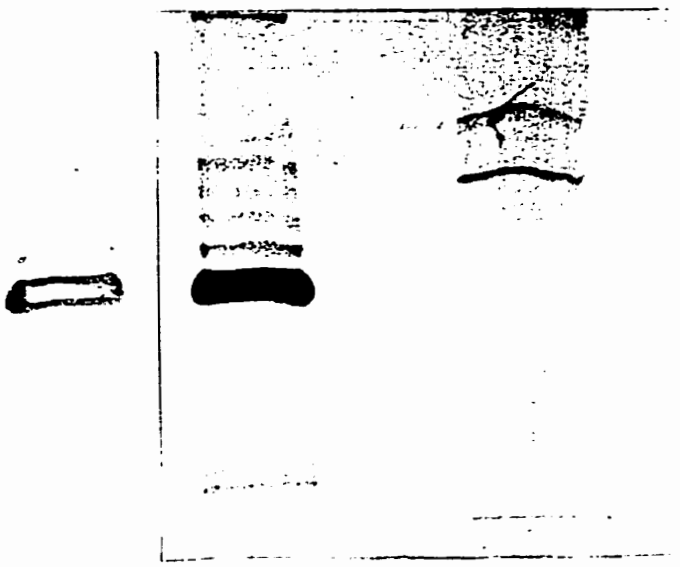
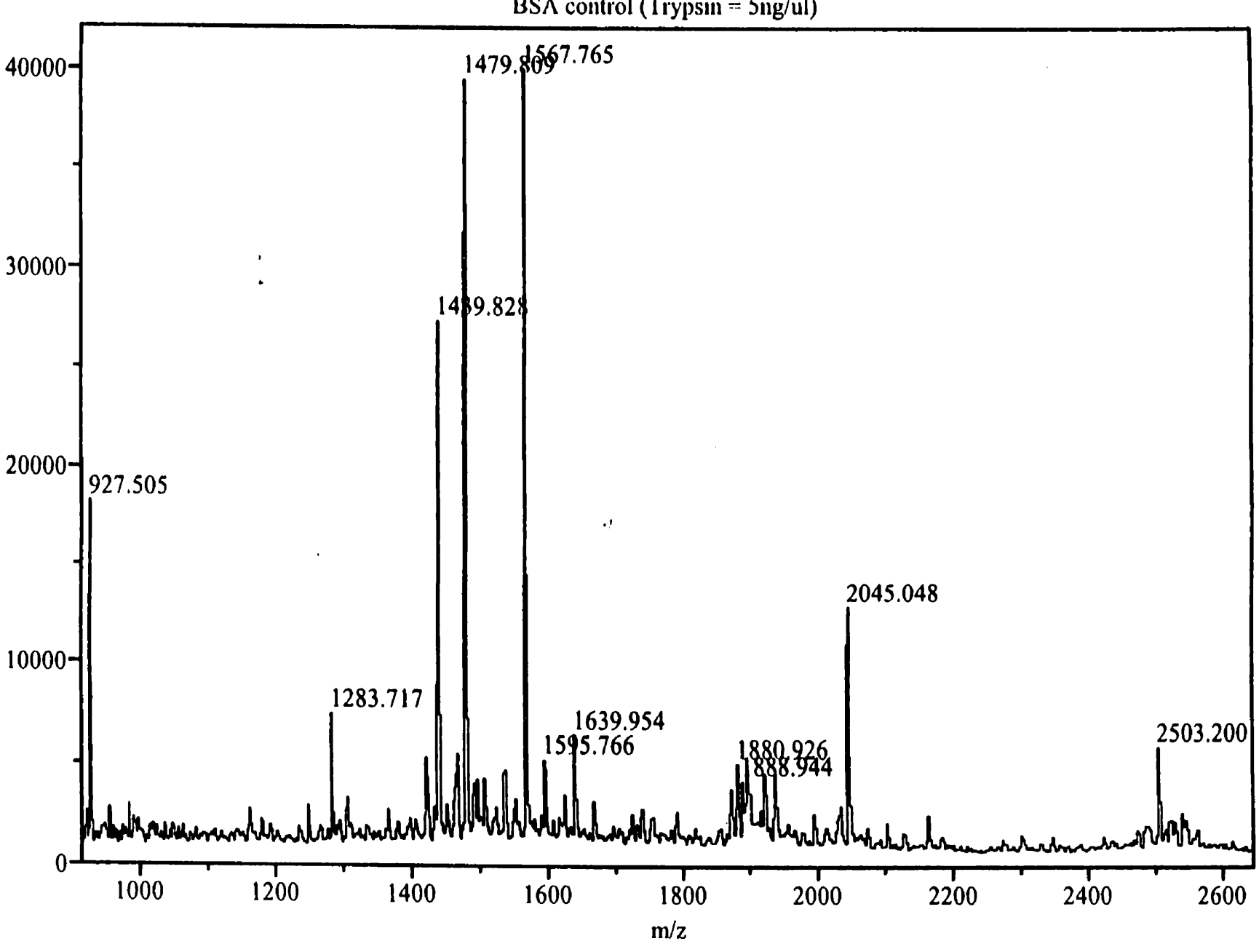


Figure 1.

Photograph of a silver-stained SDS-PAGE gel following separation of purified bovine serum albumin (BSA). Photographs were taken before and after the gel band was removed from the gel. The band of high intensity (approximately 70kDa) was cut from the gel as this band was predicted, on the basis of molecular weight and intensity, to contain the purified BSA. The various other visible bands were not cut from the gel as the focus of this experiment was on utilizing BSA alone as a positive test molecule for the established procedures.



File: b1.2calibrated, Date: 7/1/2000 11:56
Resolution: 0.625 ns, Display bin: 49.375 ns, Starts: 482869

Figure 2.

PeptIdent

Peptide mass fingerprinting

(1)	**Name given to unknown protein:**	BSA (trypsin=5ng/ul)
(2)	**Species searched:**	MAMMALIA
	Database searched:	SWISS-PROT
(3)	**pI:** 0 - ∞	
	Mw: 70000	**range:** 49000 - 91000
(4)	**Peptide masses for unknown protein:**	927.515 1249.641 1283.734 1305.728 1439.831 1479.806 1536.828 1567.762 1595.775 1871.948 1880.934 1888.960 1894.987 1921.959 1936.991 2045.047 2503.203
(5)	**Tolerance:**	±30 ppm
(6)	**Minimum number of peptides required to match:**	10
(7)	**Maximum number of matching proteins to print:**	10

(8) Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as **$[M+H]^+$**.

(9) Enzyme: **Trypsin**, allowing for **up to 2 missed cleavages (#MC).**

(10) Cysteine treated with **Iodoacetamide** to form carboxyamidomethyl cysteine (Cys_CAM), with **acrylamide adducts** (Cys_PAM).

(11) Methionine in **oxidized form.**

Scan done on 29-Feb-2000, SWISS-PROT Release 38 and updates up to 15-Feb-2000: 83857 entries.

Click here to perform a New PeptIdent Search

PeptIdent request took 6.92 CPU seconds

1 matches found.

(12) Score	(13) # peptide matches	(14) AC	(15) ID	(16) Description	(17) pI	(18) Mw
0.71	12	P02769	ALBU_BOVIN_1	CHAIN 1: SERUM ALBUMIN. - Bos taurus (Bovine).	5.60	66432.96

Figure 3.

Score: 0.71, 12 matching peptides: P02769 (ALBU_BOVIN) pI: 5.60, Mw: 66432.96
CHAIN 1: SERUM ALBUMIN. - Bos taurus (Bovine).

user mass	matching mass	Δmass (ppm)	#MC	modification	position	peptide	links
927.515	927.4934	-23.28	0		161-167	YLYEIAR	
1249.641	1249.6211	-15.91	1		35-44	FKDLGEEHFK	
1283.734	1283.7106	-18.22	0		361-371	HPEYAVSVLLR	
1305.728	1305.7161	-9.12	0		402-412	HLVDEPQNLIK	
1439.831	1439.8117	-13.39	1		360-371	RHPEYAVSVLLR	
1479.806	1479.7954	-7.16	0		421-433	LGEYGFQNALIVR	
1567.762	1567.7427	-12.31	0		347-359	DAFLGSFLYEYSR	
1880.934	1880.9211	-6.87	0	Cys_CAM: 510	508-523	RPCFSALTPDETYVPK	FindMod PeptideMass
1888.96	1888.9268	-17.57	0		169-183	HPYFYAPELLYYANK	
1888.96	1888.9949	18.49	1		89-105	SLHTLFGDELCKVASLR	BioGraph
1894.987	1894.9367	-26.53	0	Cys_PAM: 510	508-523	RPCFSALTPDETYVPK	
1921.959	1921.9364	-11.76	0	Cys_PAM: 537	529-544	LFTFHADICTLPDTEK	
1921.959	1921.9582	-0.4	2	1xCys_PAM	452-468	SLGKVGTRCCTKPESER	
2045.047	2045.0279	-9.32	1		168-183	RHPYFYAPELLYYANK	
(19)	(20)	(21)	(22)	(23)	(24)	(25)	

(26) ΔMw: 3567.0 Da (5.1%)
(27) 25.4% of sequence covered:

(28)

```
          1           11          21          31          41          51
          |           |           |           |           |           |
     1                                dthkse iahrFKDLGE EHFKglvlia fsqylqqcpf    60
    61 dehvklvnel tefaktcvad eshagcekSL HTLFGDELCK VASLRetygd madccekqep   120
   121 ernecflshk ddspdlpklk pdpntlcdef kadekkfwgk YLYEIARRHP YFYAPELLYY   180
   181 ANKyngvfqe ccqaedkgac llpkietmre kvlassarqr lrcasiqkfg eralkawsva   240
   241 rlsqkfpkae fvevtklvtd ltkvhkecch gdllecaddr adlakyicdn qdtissklke   300
   301 ccdkplleks hciaevekda ipenlpplta dfaedkdvck nyqeakDAFL GSFLYEYSRR   360
   361 HPEYAVSVLL Rlakeyeatl eeccakddph acystvfdkl kHLVDEPQNL IKqncdqfek   420
   421 LGEYGFQNAL IVRytrkvpq vstptlvevs rSLGKVGTRC CTKPESERmp ctedylslil   480
   481 nrlcvlhekt pvsekvtkcc teslvnrRPC FSALTPDETY VPKafdekLF TFHADICTLP   540
   541 DTEKqikkqt alvellkhkp kateeqlktv menfvafvdk ccaaddkeac favegpklvv   600
   601 stqtala
```

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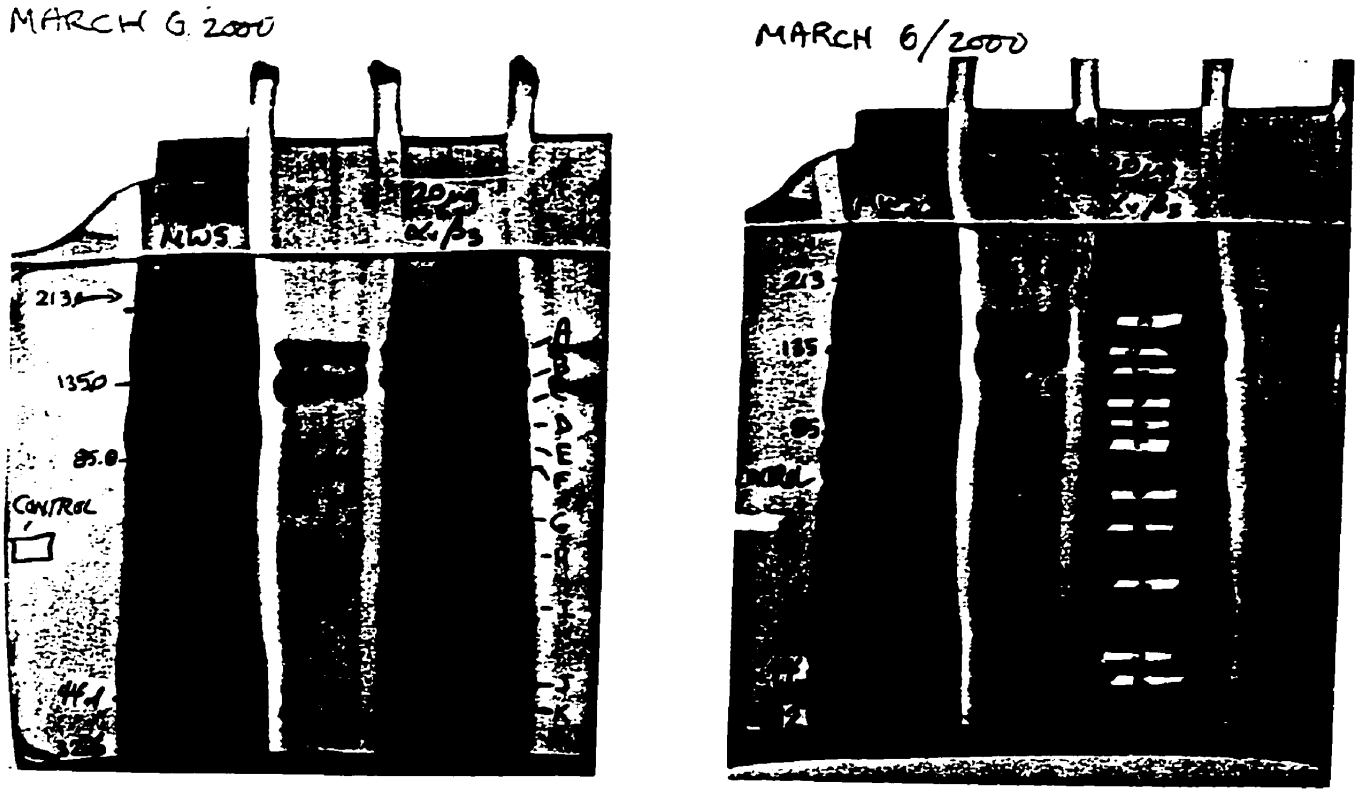
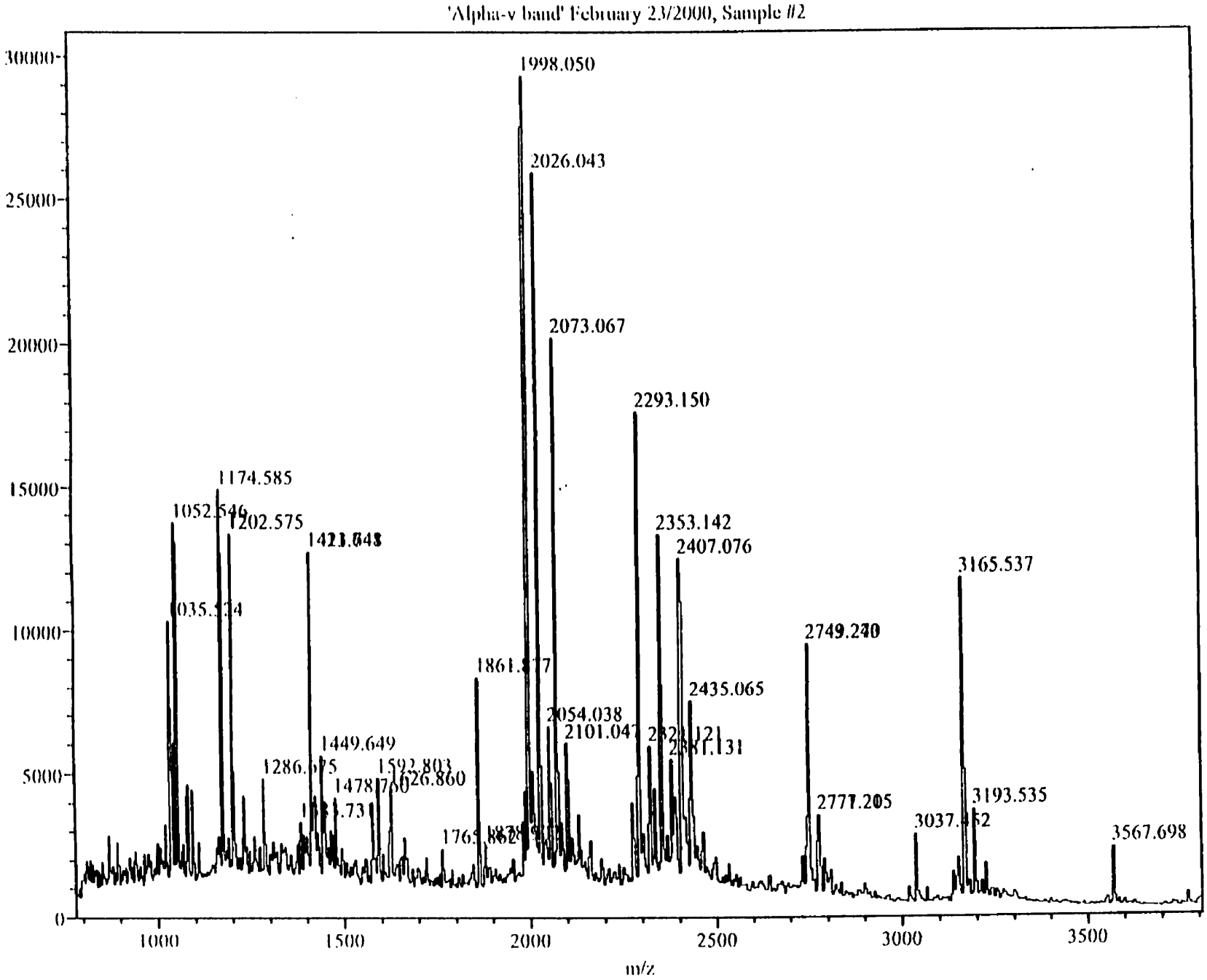


Figure 4.

Photograph (left) of a purified alphaV/beta3 preparation following separation on an SDS-PAGE gel and silver-staining. Molecular weight standards (MWS) range from 213-32.6 kDa. A total of 20ug of purified alphaV/beta3 was run and obvious bands were chosen for excision and further characterization by mass spectrometry.

Photograph (right) the same SDS-PAGE gel following band excision. The excised bands were labelled accordingly and the respective bands were then labelled on the photograph of the intact gel for accurate record keeping. Proteins were further extracted from individual gel pieces in preparation for mass spectral analysis.


'Alpha-v band' February 23/2000, Sample #2
30000
25000
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15000
10000
5000
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1998.050
2026.043
2073.067
2293.150
1174.585
1052.546
1202.575
2353.142
2407.076
3165.537
2749.240
2435.065
2054.038
2101.047
1449.649
1286.675
1592.803
1478.731
1626.860
2777.205
3193.535
3037.452
3567.698
1000
1500
2000
2500
3000
3500
m/z

Figure 5.

PeptIdent

Peptide mass fingerprinting

Name given to unknown protein:	Feb23,2000,A
Species searched:	HOMO SAPIENS (HUMAN)
Database searched:	SWISS-PROT
pI: 0 - ∞	
Mw: 130000	**range:** 0 - 260000
Peptide masses for unknown protein:	894.342 923.427 925.372 1009.461 1035.506 1038.551 1052.534 1066.546 1080.531 1092.527 1174.564 1202.556 1230.548 1259.545 1286.659 1383.703 1390.647 1399.713 1413.724 1421.629 1449.629 1465.679 1493.680 1577.729 1592.791 1605.740 1626.831 1861.848 1878.854 1998.021 2007.940 2025.997 2054.004 2073.030 2101.015 2163.026
Tolerance:	±30 ppm
Minimum number of peptides required to match:	12
Maximum number of matching proteins to print:	10

Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as $[M+H]^+$.

Enzyme: **Trypsin**, allowing for **up to 2 missed cleavages (#MC).**

Cysteine treated with **Iodoacetamide** to form carboxyamidomethyl cysteine (Cys_CAM), with **acrylamide adducts** (Cys_PAM).

Methionine in **oxidized form.**

Scan done on 25-Feb-2000, SWISS-PROT Release 38 and updates up to 15-Feb-2000: 83857 entries.

Figure 6.

Score	# peptide matches	AC	ID	Description	pI	Mw
0.47	17	P06756	ITAV_HUMAN_1	CHAIN 1: VITRONECTIN RECEPTOR ALPHA SUBUNIT. - Homo sapiens (Human).	5.21	112715.69
0.47	17	P06756	ITAV_HUMAN_2	CHAIN 2: HEAVY CHAIN. - Homo sapiens (Human).	5.11	94874.05

Score: 0.47, 17 matching peptides: P06756 (ITAV_HUMAN) pI: 5.21, Mw: 112715.69
CHAIN 1: VITRONECTIN RECEPTOR ALPHA SUBUNIT. - Homo sapiens (Human).

user mass	matching mass	Δmass (ppm)	#MC	modification	position	peptide	links
925.372	925.3832	12.16	0	Cys_PAM: 89	89-95	CDWSSTR	
1009.461	1009.4771	15.99	0	Cys_PAM: 172	166-173	TVEYAPCR	
1038.551	1038.5731	21.26	0		401-409	GIVYIFNGR	
1052.534	1052.5523	17.42	0		361-369	LNGFEVFAR	
1174.564	1174.5752	9.54	0		120-129	SHQWFGASVR	
1286.659	1286.6714	9.64	0		823-832	AMLHLQWPYK	
1383.703	1383.7341	22.46	0		582-592	LTPITIFMEYR	
1399.713	1399.729	11.42	0	MSO: 589	582-592	LTPITIFMEYR	
1399.713	1399.7303	12.37	0	Cys_CAM: 138	135-145	ILACAPLYHWR	FindMod
1413.724	1413.746	15.54	0	Cys_PAM: 138	135-145	ILACAPLYHWR	PeptideMass
1421.629	1421.6478	13.21	0	Cys_PAM: 97	97-108	CQPIEFDATGNR	BioGraph
1465.679	1465.6991	13.74	0	Cys_PAM: 158	153-165	EPVGTCFLQDGTK	
1577.729	1577.7489	12.61	1	Cys_PAM: 97	96-108	RCQPIEFDATGNR	
1592.791	1592.8067	9.85	0		447-461	NGYPDLIVGAFGVDR	
1626.831	1626.856	15.35	1		580-592	DKLTPITIFMEYR	
1878.854	1878.9014	25.25	1	Cys_PAM: 158	150-165	QEREPVGTCFLQDGTK	
1998.021	1998.0403	9.65	0		410-428	STGLNAVPSQILEGQWAAR	
2073.03	2073.0552	12.15	0		776-793	GVSSPDHIFLPIPNWEHK	

ΔMw: 17284.3 Da (13.3%)
15.5% of sequence covered.

ProFound

http://prowl.rockefeller.edu/cgi-bin/ProFound

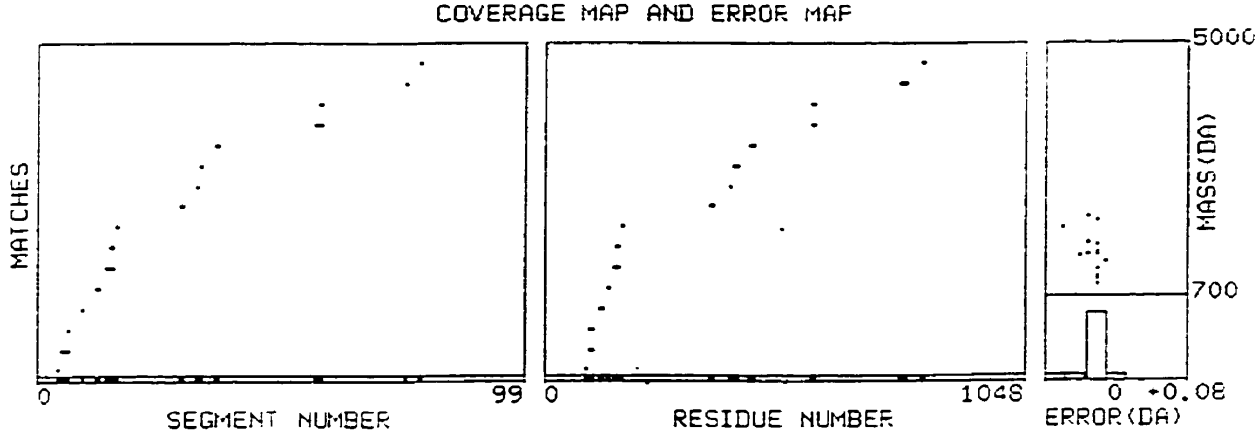
Protein identification by comparison of a peptide map to NCBI's nr database using a Bayesian algorithm.

Details for candidate 1

gi|4504763|ref|NP_002201.1| integrin alpha V precursor
gi|340307|(M14648) vitronectin alpha subunit precursor [Homo sapiens]
gi|88996|pir||A27421 integrin alpha-V chain precursor - human
gi|124959|sp|P06756|ITAV_HUMAN VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51)

```
Sample ID                    : Feb23,2000,A alpha-v band.
Digestion chemistry          : Trypsin
Number of measured peptides  : 36
Number of matched peptides   : 16
Coverage of protein sequence: 15 %
```

COVERAGE MAP AND ERROR MAP



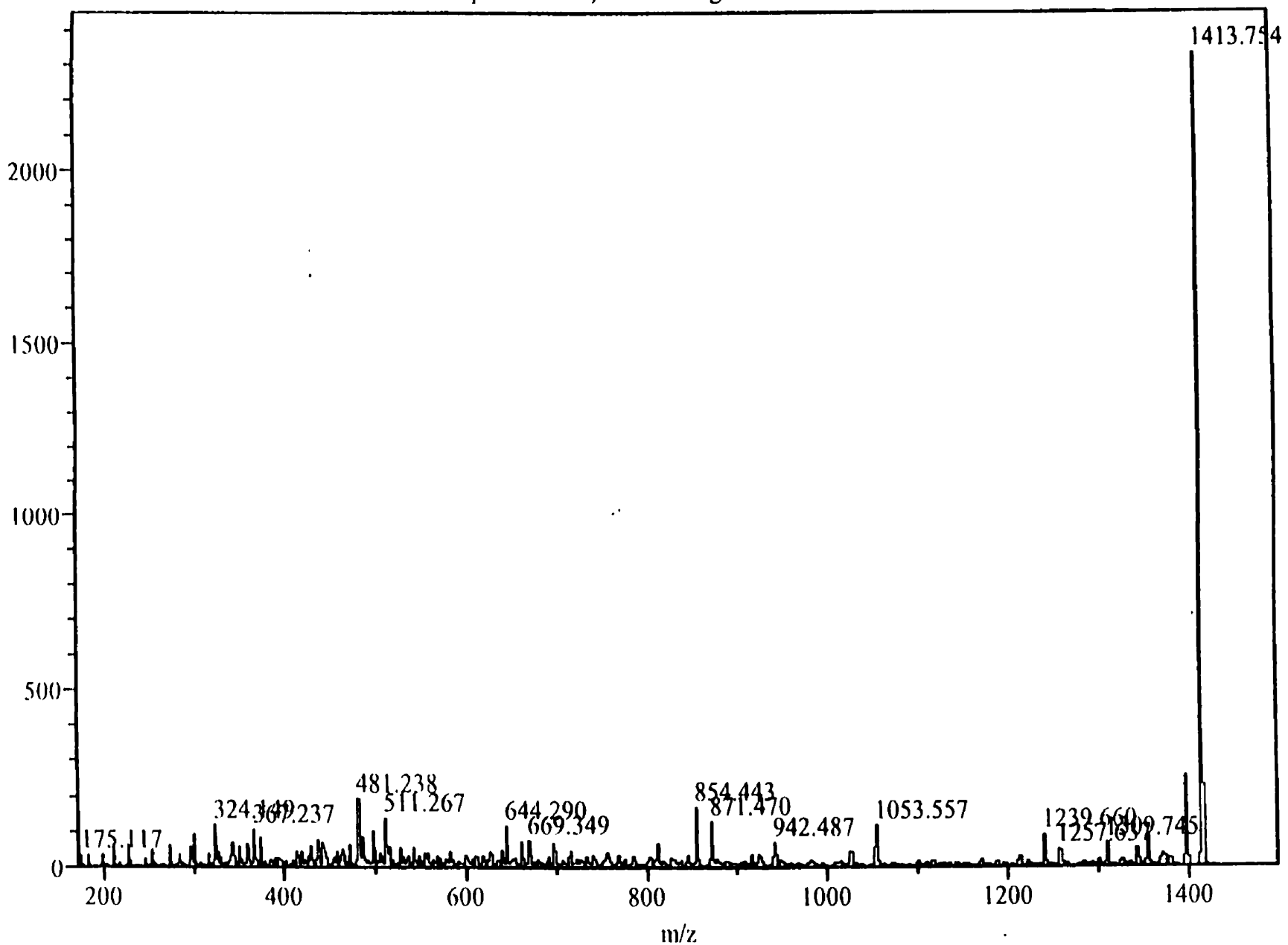
Matched Fragments Sorted by Measured Mass

Measured Mass (M)	Avg/ Mono	Computed Mass	Error (Da)	Residues From	To	Missed Cut	Cys	Met	Tyr/Trp
924.36	M	924.39	-0.02	89	95	0	1	0	1
1008.45	M	1008.47	-0.02	166	173	0	1	0	1
1037.84	M	1037.86	-0.02	401	409	0	0	0	1
1051.52	M	1051.54	-0.02	361	369	0	0	0	0
1173.55	M	1173.57	-0.02	120	129	0	0	0	1
1285.65	M	1285.66	-0.01	623	632	0	0	1	2
1382.69	M	1382.73	-0.04	582	592	0	0	1	1
1412.71	M	1412.74	-0.03	135	145	0	1	0	2
1420.62	M	1420.64	-0.02	97	108	0	1	0	0
1464.67	M	1464.69	-0.02	153	165	0	1	0	0
1576.72	M	1576.74	-0.02	96	108	1	1	0	0
1591.78	M	1591.80	-0.02	447	461	0	0	0	1
1625.82	M	1625.85	-0.03	580	592	1	0	1	1
1877.84	M	1877.90	-0.06	150	165	1	1	0	0
1997.01	M	1997.03	-0.02	410	428	0	0	0	1
2071.02	M	2071.05	-0.03	778	793	0	0	0	1

Note: '1' in tag columns Cys, Met, Tyr/Trp denotes '>=1'.

Figure 7.

Alpha-v Band, 1413.7 Fragmentation Pattern



File: sample#4,MS2,1413.75,70-80V, Date: 26/1/2000 12:8
Resolution: 0.625 ns, Display bin: 60 ns, Starts: 1227612

Figure 8.

MS-Tag Search Results

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

Sample ID (comment): **Alpha-v band,1413.75,MS2 analysis**
Database searched: **SwissProt.2.15.2000**
Full Molecular Weight range: **84482** entries
Full pI range: **84482** entries.
Species search (**HOMO SAPIENS**) selects **5685** entries.
(1) Number of sequences passing through parent mass filter: **125**
MS-Tag search selects **1** entry.

(2) Parent mass: **1413.7500 (+/- 40.0000 ppm)**
(3) Fragment Ions used in search: **175.12, 324.15, 367.24, 481.24, 511.27, 644.29, 669.35, 854.44, 871.47, 942.49, 1053.56, 1239.66, 1257.64, 1309.74 (+/- 30.00 ppm)**
(4) 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	(5) Peptide N terminus	(6) Peptide C terminus
identity	**5**	**monoisotopic**	**Trypsin**	**2**	**acrylamide**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	SwissProt.2.15.2000 Accession #	Protein Name
1	**3/14**	**(K) ILACAPLYHWR(T)**	**1413.7465**	**0.0035**	**116052.5 / 5.45**	HUMAN	**P06756**	VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51)

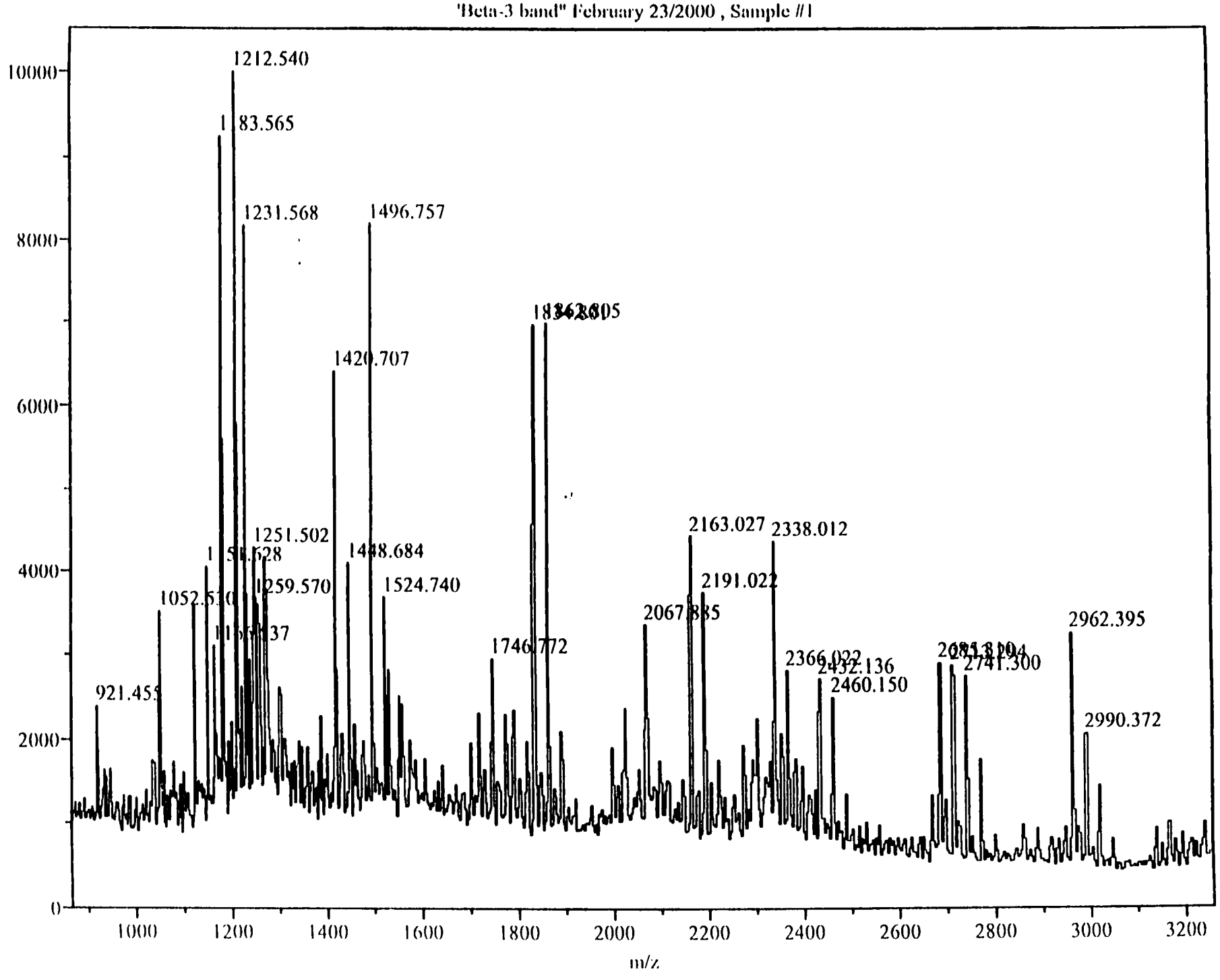
Detailed Results

Rank	(7) # Unmatched Ions	(8) Sequence	(9) MH^+ Calculated (Da)	(10) MH^+ Error (Da)	Protein MW (Da)/pI	Species	SwissProt.2.15.2000 Accession #	(11) MS-Digest Index #	Protein Name
1	**3/14**	**(K) ILACAPLYHWR(T)**	**1413.7465**	**0.0035**	**116052.5 / 5.45**	HUMAN	**P06756**	**28572**	VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51)

(12)

Fragment-ion (m/z)	175.12	324.15	367.24	481.24	511.27	644.29	669.35	854.44	871.47	942.49	1053.56	1239.66	1257.64	1309.74
Ion-type	y_1	HW		y_3-NH_3	PLYH	y_4-NH_3		y_6-NH_3	y_6	y_7	b_9	b_{10}	b_{10}+H_2O	
Delta Da	-0.00	0 00		0.01	0.00	-0.00		0.01	0.01	-0.01	0.00	0 03	-0.01	

Figure 9.


"Beta-3 band" February 23/2000 , Sample #1
10000
8000
6000
4000
2000
0
1212.540
1231.568
1496.757
1420.707
1251.502
1448.684
1524.740
1259.570
1746.772
2163.027
2338.012
2191.022
2962.395
2366.022
2460.150
2741.300
2990.372
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
3200
m/z

53

igure 10.

PeptIdent

Peptide mass fingerprinting

Name given to unknown protein:	Feb23,2000,B
Species searched:	HOMO SAPIENS (HUMAN)
Database searched:	SWISS-PROT
pI: 0 - ∞	
Mw: 130000	**range:** 0 - 260000
Peptide masses for unknown protein:	921.455 1052.530 1151.628 1166.537 1183.565 1212.540 1231.568 1251.502 1259.570 1420.707 1448.684 1496.757 1524.740 1746.772 1834.801 1862.805 2067.885 2163.027 2191.022 2338.012 2366.022 2432.136 2460.150 2685.310 2713.294 2741.300 2962.395 2990.372
Tolerance:	±30 ppm
Minimum number of peptides required to match:	9
Maximum number of matching proteins to print:	10

Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as **[M+H]$^+$**.

Enzyme: **Trypsin**, allowing for **up to 2 missed cleavages (#MC).**

Cysteine treated with **Iodoacetamide** to form carboxyamidomethyl cysteine (Cys_CAM), with **acrylamide adducts** (Cys_PAM).

Methionine in **oxidized form.**

Scan done on 25-Feb-2000, SWISS-PROT Release 38 and updates up to 15-Feb-2000: 83857 entries.

Click here to perform a New PeptIdent Search

PeptIdent request took 10.06 CPU seconds

1 matches found.

Score	# peptide matches	AC	ID	Description	pI	Mw
0.32	9	P05106	ITB3_HUMAN_1	CHAIN 1: PLATELET MEMBRANE GLYCOPROTEIN IIIA. - Homo sapiens (Human).	4.95	84517.75

Figure 11.

Score: 0.32, 9 matching peptides: P05106 (ITB3_HUMAN) pI: 4.95, Mw: 84517.75
CHAIN 1: PLATELET MEMBRANE GLYCOPROTEIN IIIA. - Homo sapiens (Human).

user mass	matching mass	Δmass (ppm)	#MC	modification	position	peptide	links
1231.568	1231.585	13.79	0	1xCys_PAM	208-217	TTCLPMFGYK	
1420.707	1420.7219	10.5	1		763-774	AKWDTANNPLYK	
1834.801	1834.8276	14.49	0	Cys_PAM: 75	73-88	DNCAPESIEFPVSEAR	
1862.805	1862.7796	-13.63	1	1xCys_PAM, 1xMSO	646-659	FDREPYMTENTCNR	FindMod
2067.885	2067.8997	7.13	0	1xCys_PAM	243-261	DAPEGGFDAIMQATVCDEK	PeptideMass
2338.012	2338.0438	13.6	1	1xCys_PAM	241-261	NRDAPEGGFDAIMQATVCDEK	BioGraph
2432.136	2432.1762	16.52	1	Cys_PAM: 75	68-88	ENLLKDNCAPESIEFPVSEAR	
2685.31	2685.3438	12.58	1		89-113	VLEDRPLSDKGSGDSSQVTQVSPQR	
2962.395	2962.4284	11.29	2	2xCys_PAM	64-88	CDLKENLLKDNCAPESIEFPVSEAR	

ΔMw: 45482.2 Da (35.0%)
14.0% of sequence covered:

ProFound

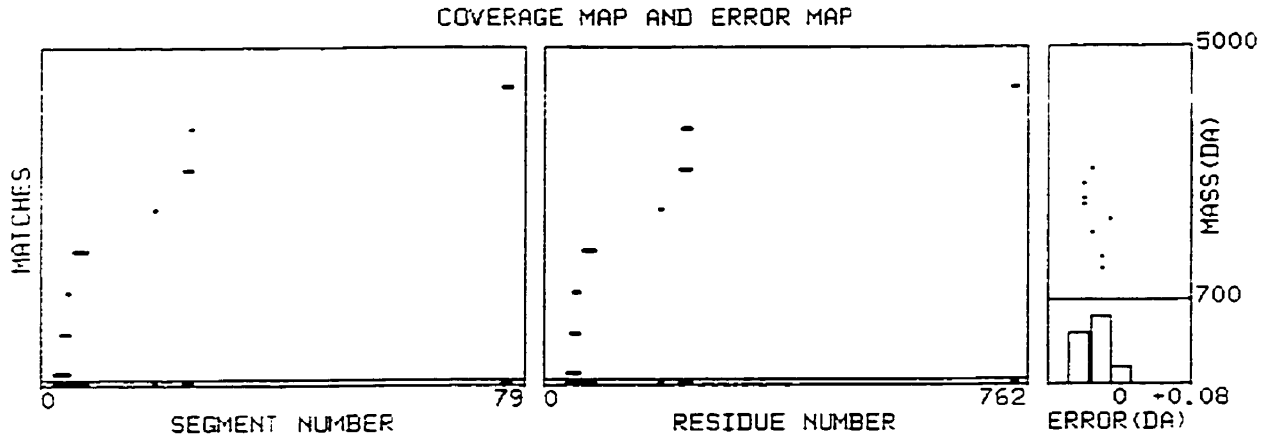
http://prowl.rockefeller.edu/cgi-bin/ProFound

Protein identification by comparison of a peptide map to NCBI's nr database using a Bayesian algorithm

Details for candidate 1

gi|497440|(M32686) platelet glycoprotein [Homo sapiens]

```
Sample ID                    : Feb23,2000,B(beta-3 band.
Digestion chemistry          : Trypsin
Number of measured peptides  : 28
Number of matched peptides   : 8
Coverage of protein sequence: 12 %
```

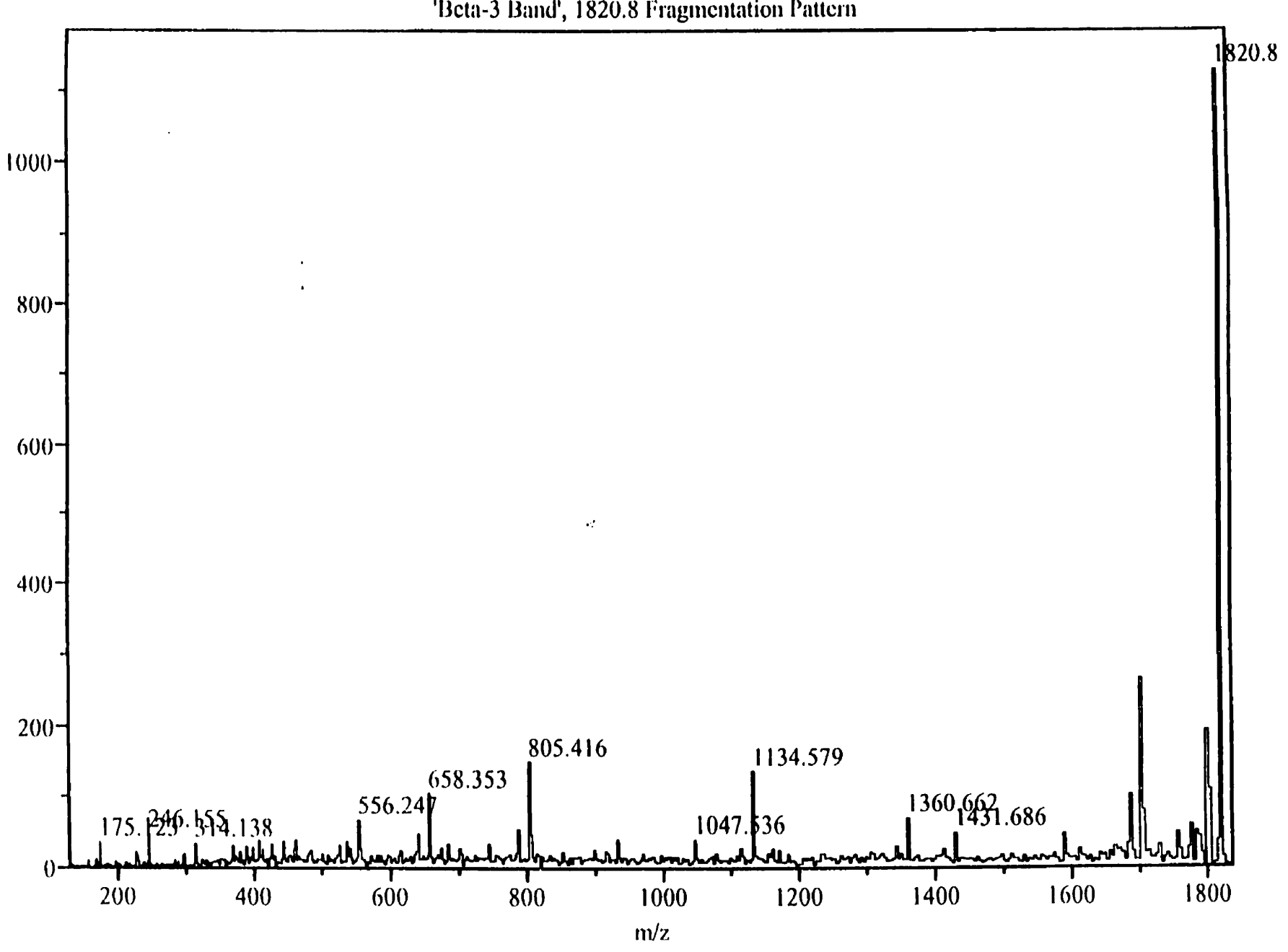


Matched Fragments Sorted by Measured Mass

Measured Mass(M)	Avg/ Mono	Computed Mass	Error (Da)	Residues From	To	Missed Cut	Cys	Met	Tyr/Trp
1230.56	M	1230.59	-0.02	182	191	0	1	1	1
1419.70	M	1419.72	-0.02	737	748	1	0	0	2
1833.79	M	1833.82	-0.03	46	61	0	1	0	0
2066.88	M	2066.89	-0.01	217	235	0	1	1	0
2337.00	M	2337.04	-0.04	218	235	1	1	1	0
2431.13	M	2431.17	-0.04	41	61	1	1	0	0
2684.30	M	2684.34	-0.04	62	86	2	0	0	0
2961.39	M	2961.42	-0.03	37	61	2	2	0	0

Note: '2' in tag columns (Cys, Met, Tyr/Trp) denotes '>=2'.

Figure 12.



File: 018, 7c, MS2, 1820.8, 95V, Date: 26/5/1999 16:26
Resolution: 0.625 ns, Display bin: 86.875 ns, Starts: 863291

Figure 13.

MS-Tag Search Results

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

Sample ID (comment): **Beta-3 band,1820.84,MS2 analysis**
Database searched: **SwissProt.2.15.2000**
Full Molecular Weight range: **84482** entries.
Full pI range: **84482** entries.
Species search (**MAMMALS**) selects **15109** entries.
Number of sequences passing through parent mass filter: **178**
MS-Tag search selects **1** entry.

Parent mass: **1820.8400 (+/- 40.0000 ppm)**
Fragment Ions used in search: **175.12, 246.16, 314.14, 556.25, 658.35, 805.42, 1047.54, 1134.58, 1360.66, 1431.69 (+/- 30.00 ppm)**
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	**4**	**monoisotopic**	**Trypsin**	**2**	**carbamidomethylation**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH+ Calculated (Da)	MH+ Error (Da)	Protein MW (Da)/pI	Species	SwissProt.2.15.2000 Accession #	MS-Digest Index #	Protein Na
1	0/10	(K) DNCAPESIEFPVSEAR(V)	1820.8125	0.0275	87214.7 / 5.07	HUMAN	P05106	28973	PLATELET MEMBRAN GLYCOPR IIIA PRECURSO (GPIIIA) (INTEGRIN BETA-3) (C

Fragment-ion (m/z)	175.12	246.16	314.14	556.25	658.35	805.42	1047.54	1134.58	1360.66	1431.69
Ion-type	y_1	y_2	PES	PESIE	y_6	y_7	y_9	y_{10}	y_{12}	y_{13}
Delta Da	0.00	-0.00	0.00	-0.01	0.00	-0.00	-0.01	-0.00	-0.01	-0.03

Figure 14.

PepFrag Search Results

Spectrum description:
Beta-3 band,1820.84,MS2 analysis
Database: sprot
Kingdom: mammals
Maximum number of proteins in result: 10
Protein mass: 0.0-3000.0 kDa
Protein pI: 0.0-15.0
Iodoacetamide
Enzyme: Trypsin, # of incompletes: 2

Mass of parent peptide: 1819.8 +/- 0.1 , charge state = 1+
Maximum number of phosphorylations per peptide: 0 S/T and 0 Y.
Fragments: 175.1, 246.2, 314.1, 556.2, 658.4, 805.4, 1047.5, 1134.6, 1360.7, 1431.7, Error: 0.1, Matches: 7
Ion types: b, b*, y", y*, Carboxypeptidase, Aminopeptidase

Searching: sprot-primates

ITB3_HUMAN PLATELET MEMBRANE GLYCOPROTEIN IIIA PRECURSOR (GPIIIA) (INTEGRIN BETA-3) (CD61) - HOMO SAPIENS (HUMAN) mass = 90349.6 Da, pI = 5.1

ENLLK**DNCAPESIEFPVSEAR**VLEDR

175.12 +/- 0.10 Da: **y"1** (175.12 Da)
246.15 +/- 0.10 Da: **y"2** (246.16 Da)
314.14 +/- 0.10 Da:
556.25 +/- 0.10 Da:
658.35 +/- 0.10 Da: **y"6** (658.35 Da)
805.42 +/- 0.10 Da: **y"7** (805.42 Da)
1047.54 +/- 0.10 Da: **y"9** (1047.55 Da)
1134.58 +/- 0.10 Da: **y"10** (1134.58 Da)
1360.66 +/- 0.10 Da: **y"12** (1360.67 Da)
1431.69 +/- 0.10 Da: **y"13** (1431.71 Da)

Not searched: sprot-rodents

Not searched: sprot-other-mammals

Search time = 1 s

PROWL

webmaster

Figure 15.

PeptIdent

Peptide mass fingerprinting

Name given to unknown protein:	All extracted peptides for alpha-v
Species searched:	HOMO SAPIENS (HUMAN)
Database searched:	SWISS-PROT
pI: 0 - ∞	
Mw: 130000	**range:** 91000 - 169000
Peptide masses for unknown protein:	869.5 925.37 979.5 999.6 1038.55 1009.46 1052.53 1174.56 1642.8 1267.65 1286.66 1247.6 1360.69 1383.75 1399.71 1413.72 1421.63 1423.7 1465.68 1488.74 1592.79 1577.73 1626.83 1652.8 1847.9 1878.85 1668.7 1998.02 2073.03 2293.17 2299.1 2353.2 2368.08 2407.08 2743.2 2749.24 2791.5 3037.54 3165.59 3567.71
Tolerance:	±40 ppm
Minimum number of peptides required to match:	20
Maximum number of matching proteins to print:	10

Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as $[M+H]^+$.

Enzyme: **Trypsin**, allowing for **up to 2 missed cleavages (#MC).**

Cysteine treated with **Iodoacetamide** to form carboxyamidomethyl cysteine (Cys_CAM), with **acrylamide adducts** (Cys_PAM).

Methionine in **oxidized form.**

Scan done on 14-Mar-2000, SWISS-PROT Release 38 and updates up to 15-Feb-2000: 83857 entries.

Click here to perform a New PeptIdent Search

PeptIdent request took 14.71 CPU seconds

2 matches found.

Score	# peptide matches	AC	ID	Description	pI	Mw
1.00	40	P06756	ITAV_HUMAN_1	CHAIN 1: VITRONECTIN RECEPTOR ALPHA SUBUNIT. - Homo sapiens (Human).	5.21	112715.69
0.88	35	P06756	ITAV_HUMAN_2	CHAIN 2: HEAVY CHAIN. - Homo sapiens (Human).	5.11	94874.05

Figure 16.

Score: 1.00, 40 matching peptides: P06756 (ITAV_HUMAN) pI: 5.21, Mw: 112715.69
CHAIN 1: VITRONECTIN RECEPTOR ALPHA SUBUNIT. - Homo sapiens (Human).

user mass	matching mass	Δmass (ppm)	#MC	modification	position	peptide	links
869.5	869.488	-13.88	0		541-547	ALFLYSR	
925.37	925.383	14.32	0	Cys_PAM: 89	89-95	CDWSSTR	
979.5	979.496	-4.53	0		234-241	YNNQLATR	
999.6	999.595	-5.48	0		719-728	AGTQLLAGLR	
1038.55	1038.573	22.23	0		401-409	GIVYIFNGR	
1009.46	1009.477	16.99	0	Cys_PAM: 172	166-173	TVEYAPCR	
1052.53	1052.552	21.23	0		361-369	LNGFEVFAR	
1174.56	1174.575	12.94	0		120-129	SHQWFGASVR	
1642.8	1642.851	30.98	1	MSO: 589	580-592	DKLTPITIFMEYR	
1267.65	1267.639	-8.75	0		1024-1033	VRPPQEEQER	
1286.66	1286.671	8.87	0		823-832	AMLHLQWPYK	
1247.6	1247.648	38.3	1		636-646	LEVSVDSDQKK	
1360.69	1360.664	-19.29	1	MSO: 555	548-559	SPSHSKNMTISR	
1383.75	1383.734	-11.52	0		582-592	LTPITIFMEYR	
1399.71	1399.729	13.56	0	MSO: 589	582-592	LTPITIFMEYR	
1399.71	1399.73	14.51	0	Cys_CAM: 138	135-145	ILACAPLYHWR	
1413.72	1413.746	18.38	0	Cys_PAM: 138	135-145	ILACAPLYHWR	
1421.63	1421.648	12.51	0	Cys_PAM: 97	97-108	CQPIEFDATGNR	
1423.7	1423.74	28.13	1		1023-1033	RVRPPQEEQER	
1465.68	1465.699	13.06	0	Cys_PAM: 158	153-165	EPVGTCFLQDGTK	
1488.74	1488.737	-2.08	0		952-964	SSASFNVIEFPYK	
1592.79	1592.807	10.48	0		447-461	NGYPDLIVGAFGVDR	FindMod
1577.73	1577.749	11.97	1	Cys_PAM: 97	96-108	RCQPIEFDATGNR	PeptideMass
1626.83	1626.856	15.97	1		580-592	DKLTPITIFMEYR	
1652.8	1652.813	8.14	0	1xCys_CAM	560-573	GGLMQCEELIAYLR	BioGraph
1847.9	1847.929	15.96	1	1xMSO	135-149	ILACAPLYHWRTEMK	
1878.85	1878.901	27.38	1	Cys_PAM: 158	150-165	QEREPVGTCFLQDGT K	
1668.7	1668.71	5.74	0		1034-1048	EQLQPHENGEGNSET	
1998.02	1998.04	10.15	0		410-428	STGLNAVPSQILEGQ WAAR	

PeptIdent

2073.03	2073.055	12.15	0		776-793	GVSSPDHIFLPIPNW EHK
2293.17	2293.146	-10.53	1		440-461	GATDIDKNGYPDLIV GAFGVDR
2299.1	2299.153	23.18	1		890-911	RDLALSEGDIHTLGC GVAQCLK
2353.2	2353.131	-29.5	0		794-813	ENPETEEDVGPVVQH IYELR
2368.08	2368.127	19.91	2	1xCys_PAM	146-165	TEMKQEREPVGTCFL QDGTK
2407.08	2407.051	-12.19	0	Cys_PAM: 185	174-195	SQDIDADGQGFCQGG FSIDFTK
2743.2	2743.267	24.35	2	Cys_PAM: 97	97-119	CQPIEFDATGNRDYA KDDPLEFK
2749.24	2749.216	-8.8	1	Cys_PAM: 185	174-198	SQDIDADGQGFCQGG FSIDFTKADR
2791.5	2791.466	-12.02	0		199-224	VLLGGPGSFYWQGQL ISDQVAEIVSK
3037.54	3037.442	-32.12	0		370-399	FGSAIAPLGDLDQDG FNDIAIAAPYGGEDK
3165.59	3165.537	-16.61	1		370-400	FGSAIAPLGDLDQDG FNDIAIAAPYGGEDK K
3567.71	3567.655	-15.42	0		242-275	TAQAIFDDSYLGYSV AVGDFNGDGIDDFVS GVPR

ΔMw: 17284.3 Da (13.3%)
43.1% of sequence covered:

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    .                                   fnldvdspae ysgpegsyfg favdffvpsa     60
   61 ssrmfllvga pkanttqpgl vegggvlkCD WSSTRRCQPI EFDATGNRDY AKDDPLEFKS    120
  121 HQWFGASVRs kqdkILACAP LYHWRTEMKQ EREPVGTCFL QDGTKTVEYA PCRSQDIDAD    180
  181 GQGFCQGGFS IDFTKADRVL LGGPGSFYWQ GQLISDQVAE IVSKydpnvy sikYNNQLAT    240
  241 RTAQAIFDDS YLGYSVAVGD FNGDGIDDFV SGVPRaartl gmvyiydgkn mssiynftge    300
  301 qmaayfgfsv aatdingddy advfigaplf mdrgsdgklq evgqvsvslq rasgdfqttk    360
  361 LNGFEVFARF GSAIAPLGDL DQDGFNDIAI AAPYGGEDKK GIVYIFNGRS TGLNAVPSQI    420
  421 LEGQWAARsm ppsfgysmkG ATDIDKNGYP DLIVGAFGVD Railyrarpv itvnaglevy    480
  481 psilnqdnkt cslpqtalkv scfnvrfclk adgkgvlprk lnfqvellld klkqkgairr    540
  541 ALFLYSRSPS HSKNMTISRG GLMQCEELIA YLRdesefrD KLTPITIFME YRldyrtaad    600
  601 ttglqpilnq ftpanisrqa hilldcgedn vckpkLEVSV DSDQKKiyiq ddnpltlivk    660
  661 aqnqgegaye aeiivsiplq adfigvvrnn ealarlscaf ktenqtrqvv cdlgnpmkAG    720
  721 TQLLAGLRfs vhqqsemdts vkfdlqlqss nlfdkvspvv shkvdlavla aveirGVSSP    780
  781 DHIFLPIPNW EHKENPETEE DVGPVVQHIY ELRnngpssf skAMLHLQWP YKynnntlly    840
  841 tlnyqlqgpm nctsameinp lrikissiqt teknatvaqg gerdhlitkR DLALSEGDIH    900
  901 TLGCGVAQCL Klvcqvgrlc rgksaillyvk sllwtetfmn kenqnnsysi kSSASFNVIE    960
  961 FPYKnlpiec itnstlvttn vtwglqpapm pvpvwvilla vlagllllav lvfvmyrmgf   1020
 1021 fKRVRPPQEE QEREQLQPHE NGEGNSET
```

PeptIdent

Peptide mass fingerprinting

Name given to unknown protein:	All extracted Beta-3 peptides
Species searched:	HOMO SAPIENS (HUMAN)
Database searched:	SWISS-PROT
pI: 0 - ∞	
Mw: 100000	**range:** 70000 - 130000
Peptide masses for unknown protein:	709.3 882.6 893.5 959.55 980.55 1060.505 1123.63 1160.572 1184.6 1217.6 1223.561 1231.6 1266.686 1282.7 1389.7 1420.7 1531.8 1552.6 1510.572 1729.823 1763.827 1791.766 1795.811 1820.847 1862.8 1834.8 2023.07 2067.934 2283.0 2289.0 2338.0 2418.2 2432.2 2685.4 2962.4
Tolerance:	±40 ppm
Minimum number of peptides required to match:	20
Maximum number of matching proteins to print:	10

Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as **[M+H]$^+$**.

Enzyme: **Trypsin**, allowing for **up to 2 missed cleavages (#MC).**

Cysteine treated with **Iodoacetamide** to form carboxyamidomethyl cysteine (Cys_CAM), with **acrylamide adducts** (Cys_PAM).

Methionine in **oxidized form**.

Scan done on 20-Mar-2000, SWISS-PROT Release 38 and updates up to 15-Feb-2000: 83857 entries.

Click here to perform a New PeptIdent Search

PeptIdent request took 11.10 CPU seconds

1 matches found.

Score	# peptide matches	AC	ID	Description	pI	Mw
0.97	34	P05106	ITB3_HUMAN_1	CHAIN 1: PLATELET MEMBRANE GLYCOPROTEIN IIIA. - Homo sapiens (Human).	4.95	84517.75

Figure 17.

Score: 0.97, 34 matching peptides: P05106 (ITB3_HUMAN) pI: 4.95, Mw: 84517.75
CHAIN 1: PLATELET MEMBRANE GLYCOPROTEIN IIIA. - Homo sapiens (Human).

user mass	matching mass	Δmass (ppm)	#MC	modification	position	peptide	links
709.3	709.315	21.35	0		756-760	FEEER	
893.5	893.473	-30.6	1		229-235	FNEEVKK	
959.55	959.552	2.08	1		379-386	SKVELEVR	
980.55	980.589	39.52	0		743-750	LLITIHDR	
1060.505	1060.484	-19.81	0	Cys_CAM: 521	516-524	EGQPVCSQR . .	
1123.63	1123.651	18.68	0		439-448	SFTIKPVGFK	
1160.572	1160.548	-20.82	0		208-217	TTCLPMFGYK	
1184.6	1184.558	-35.31	1		752-760	EFAKFEEER	
1217.6	1217.569	-25.22	0	1xCys_CAM	208-217	TTCLPMFGYK	
1223.561	1223.521	-32.31	0		693-702	FQYYEDSSGK	
1231.6	1231.585	-12.2	0	1xCys_PAM	208-217	TTCLPMFGYK	
1266.686	1266.658	-22.45	0		417-428	IGDTVSFSIEAK	
1282.7	1282.711	8.86	0		218-228	HVLTLTDQVTR	
1389.7	1389.701	0.6	0		152-163	DDLWSIQNLGTK	
1420.7	1420.722	15.43	1		763-774	AKWDTANNPLYK	
1531.8	1531.775	-16.28	0		266-279	NDASHLLVFTTDAK	
1552.6	1552.587	-8.68	0	3xCys_PAM	546-556	YCECDDFSCVR	
1510.572	1510.54	-21.49	0	3xCys_CAM	546-556	YCECDDFSCVR	
1729.823	1729.767	-32.62	1		590-606	TDTCMSSNGLLCSGR GK	
1763.827	1763.79	-20.72	0		73-88	DNCAPESIEFPVSEA R	FindMod
1791.766	1791.742	-13.12	1	1xMSO	646-659	FDREPYMTENTCNR	PeptideMass
1795.811	1795.745	-36.89	1	1xCys_CAM	542-556	ITGKYCECDDFSCVR	
1820.847	1820.812	-19.27	0	Cys_CAM: 75	73-88	DNCAPESIEFPVSEA R	BioGraph
1862.8	1862.78	-10.95	1	1xCys_PAM, 1xMSO	646-659	FDREPYMTENTCNR	
1834.8	1834.828	15.03	0	Cys_PAM: 75	73-88	DNCAPESIEFPVSEA R	
2023.07	2023.068	-0.95	2		431-448	GCPQEKEKSFTIKPV GFK	
2067.934	2067.9	-16.57	0	1xCys_PAM	243-261	DAPEGGFDAIMQATV CDEK	
2283	2283.002	0.69	1	1xMSO	241-261	NRDAPEGGFDAIMQA TVCDEK	
2289	2288.984	-6.95	2	4xCys_PAM	627-644	CPTCPDACTFKKECV ECK	

PeptIdent

2338	2337.928	-30.94	1	1xCys_PAM	605-626	GKCECGSCVCIQPGS YGDTCEK
2338	2338.044	18.73	1	1xCys_PAM	241-261	NRDAPEGGFDAIMQA TVCDEK
2418.2	2418.161	-16.33	1	Cys_CAM: 75	68-88	ENLLKDNCAPESIEF PVSEAR
2432.2	2432.176	-9.79	1	Cys_PAM: 75	68-88	ENLLKDNCAPESIEF PVSEAR
2685.4	2685.344	-20.93	1		89-113	VLEDRPLSDKGSGDS SQVTQVSPQR
2962.4	2962.428	9.6	2	2xCys_PAM	64-88	CDLKENLLKDNCAPE SIEFPVSEAR

ΔMw: 15482.2 Da (15.5%)
38.7% of sequence covered:

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1                              gpnl cttrgvsscq qclavspmca wcsdealplg    60
   61 sprCDLKENL LKDNCAPESI EFPVSEARVL EDRPLSDKGS GDSSQVTQVS PQRialrlrp   120
  121 ddsknfsiqv rqvedypvdi yylmdlsysm kDDLWSIQNL GTKlatqmrk ltsnlrigfg   180
  181 afvdkpvspy myisppeale npcydmkTTC LPMFGYKHVL TLTDQVTRFN EEVKKqsvsr   240
  241 NRDAPEGGFD AIMQATVCDE KigwrNDASH LLVFTTDAKt hialdgrlag ivqpndgqch   300
  301 vgsdnhysas ttmdypslgl mteklsqkni nlifavtenv vnlyqnysel ipgttvgvls   360
  361 mdssnvlqli vdaygkirSK VELEVRdlpe elslsfnatc innevipglk scmglkIGDT   420
  421 VSFSIEAKvr GCPQEKEKSF TIKPVGFKds livqvtfdcd cacqaqaepn shrcnngngt   480
  481 fecgvcrcgp gwlgsqcecs eedyrpsqqd ecsprEGQPV CSQRgeclcg qcvchssdfg   540
  541 kITGKYCECD DFSCVRykge mcsghgqcsc gdclcdsdwt gyycncttrT DTCMSSNGLL   600
  601 CSGRGKCECG SCVCIQPGSY GDTCEKCPTC PDACTFKKEC VECKkFDREP YMTENTCNRy   660
  661 crdelesvke lkdtgkcavn ctykneddcv vrFQYYEDSS GKsilyvvee pecpkgpdil   720
  721 vvllsvmgai lliglaalli wkLLITIHDR kEFAKFEEER arAKWDTANN PLYKeatstf   780
  781 tnityrgt
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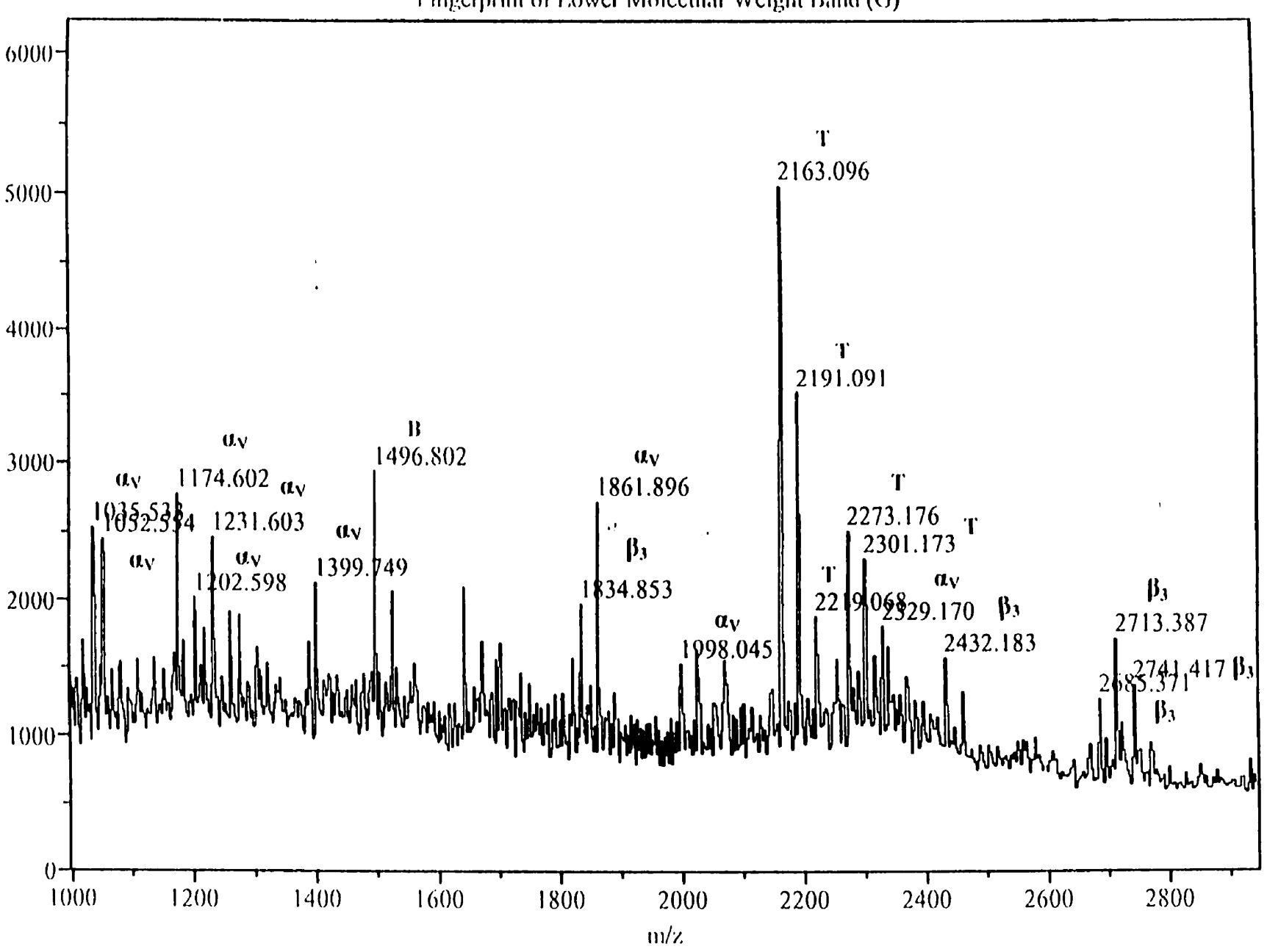


File: #8,ms, Date: 18/1/2000 14:43
Resolution: 0.625 ns, Display bin: 52.5 ns, Starts: 369189

Figure 18.

PepFrag Search Results

Spectrum description:
March 8/2000 I (unknown) 1954.09
Database: sprot
Kingdom: primates
Maximum number of proteins in result: 10
Protein mass: 0.0-150.0 kDa
Protein pI: 0.0-15.0
Acrylamide
Enzyme: Trypsin, # of incompletes: 2

Mass of parent peptide: 1953.1 +/- 0.1 , charge state = 1+
Maximum number of phosphorylations per peptide: 0 S/T and 0 Y.
Fragments: 310.2, 325.2, 356.1, 465.2, 493.2, 568.4, 635.3, 663.3, 689.4, 724.4, 760.4, 802.4, 831.5, 859.5, 944.5, 972.5, 1085.6, 1168.7, 1216.7, 1291.8, 1315.7, 1386.8, 1540.9, 1711.0, Error: 0.1,
Matches: 14
Ion types: a, a*, b, b*, y'', y*, Carboxypeptidase, Aminopeptidase

Searching: sprot-primates

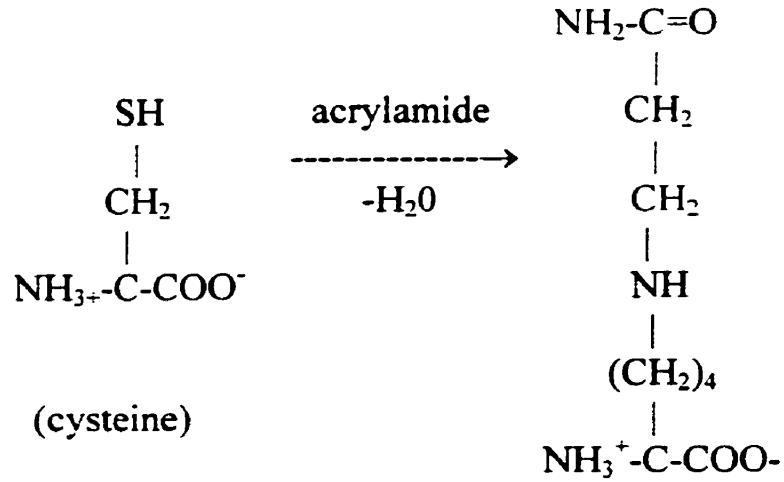
ACTB_HUMAN ACTIN, CYTOPLASMIC 1 (BETA-ACTIN) - HOMO SAPIENS (HUMAN), MUS MUSCULUS (MOUSE), RATTUS NORVEGICUS (RAT), BOS TAURUS (BOVINE), AND GALLUS GALLUS (CHICKEN) mass = 42135.9 Da, pI = 5.3

YNELRV**APEEHPVLLTEAPLNPK**ANREK

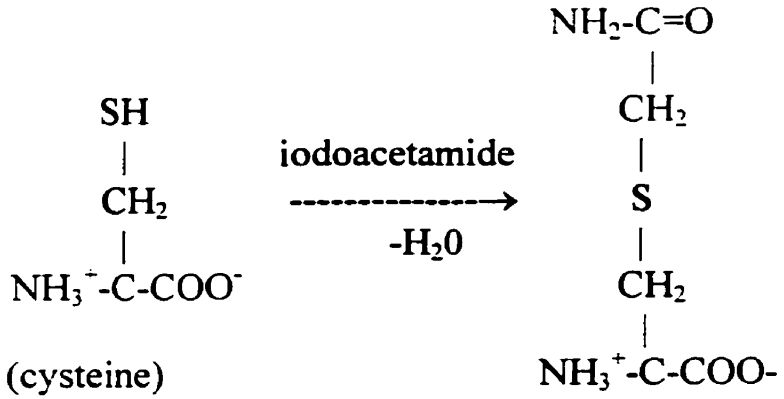
310.21 +/- 0.10 Da:
325.20 +/- 0.10 Da:
356.15 +/- 0.10 Da:
465.22 +/- 0.10 Da:
493.22 +/- 0.10 Da:
568.36 +/- 0.10 Da: **y''5** (568.35 Da)
635.34 +/- 0.10 Da: **a6** (635.32 Da)
663.33 +/- 0.10 Da: **b6** (663.31 Da)
689.37 +/- 0.10 Da:
724.45 +/- 0.10 Da:
760.40 +/- 0.10 Da: **b7** (760.36 Da)
802.45 +/- 0.10 Da:
831.48 +/- 0.10 Da: **a8** (831.44 Da)
859.45 +/- 0.10 Da: **b8** (859.43 Da)
944.55 +/- 0.10 Da: **a9** (944.52 Da)
972.54 +/- 0.10 Da: **b9** (972.51 Da)
1085.61 +/- 0.10 Da: **b10** (1085.60 Da)
1168.70 +/- 0.10 Da:
1216.66 +/- 0.10 Da:
1291.82 +/- 0.10 Da: **y''12** (1291.76 Da)
1315.72 +/- 0.10 Da: **b12** (1315.69 Da)
1386.78 +/- 0.10 Da: **b13** (1386.73 Da)
1540.86 +/- 0.10 Da: **y*14** (1540.86 Da)
1710.97 +/- 0.10 Da: **b16** (1710.91 Da)

Figure 19.

CARBAMIDOETHYLATION



CARBAMIDOMETHYLATION



FORMYLATION

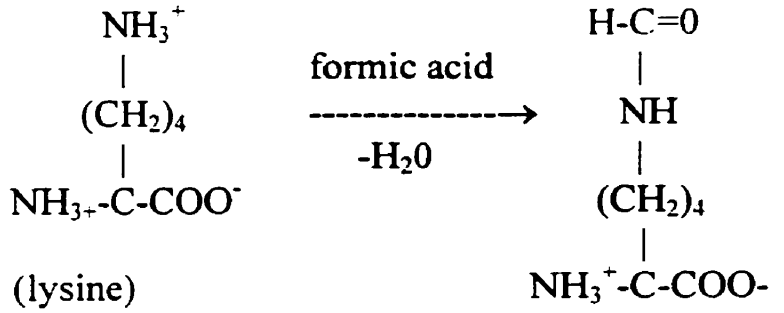


Figure 20.

Chemical treatment of peptides results in the respective chemical modifications illustrated. Lysine is an example of a basic amino acid.

MS-Tag Search Results

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

Sample ID (comment): **Feb2/2000,Alpha-v band(#4),,ms2,2381.2**
Database searched: **SwissProt.2.15.2000**
Full Molecular Weight range: **84482** entries.
Full pI range: **84482** entries.
Species search (**HUMAN MOUSE**) selects **9303** entries.
Number of sequences passing through parent mass filter: **179**
MS-Tag search selects **1** entry.

Parent mass: **2353.2000 (+/- 40.0000 ppm)**
Fragment Ions used in search: **266.01, 288.19, 379.22, 498.17, 580.32, 599.23, 618.36, 710.25, 813.46, 830.45, 1122.70, 1253.63, 1310.71, 1409.79, 1524.80, 1689.70, 1883.96, 2093.92, 2110.05, 2207.04 (+/- 40.00 ppm)**
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	**13**	**monoisotopic**	**Trypsin**	**2**	**acrylamide**	**Hydrogen (H)**	**Free Acid (O H)**

Result Summary

Rank	# Unmatched Ions	Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	SwissProt.2.15.2000 Accession #	Protein Name
1	**8/20**	**(K) ENPETEEDVGPVVQHIYELR(N)**	**2353.1312**	**0.0688**	**116052.5 / 5.45**	HUMAN	**P06756**	VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51)

Figure 21.

PepFrag Search Results

Spectrum description:
Alpha-v peptide 1052.58 (unmodified)
Database: sprot
Kingdom: primates
Maximum number of proteins in result: 10
Protein mass: 0.0-300.0 kDa
Protein pI: 0.0-15.0
Acrylamide
Enzyme: Trypsin, # of incompletes: 2

Mass of parent peptide: 1051.6 +/- 0.1 , charge state = 1+
Maximum number of phosphorylations per peptide: 0 S/T and 0 Y.
Fragments: 175.1, 334.1, 387.2, 431.2, 448.2, 492.3, 544.2, 561.3, 643.3, 660.3, 825.4, 922.4. Error: 0.1, Matches: 7
Ion types: a, a*, b, b*, y", y*. Carboxypeptidase. Aminopeptidase

Searching: sprot-primates

ITAV_HUMAN VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51) - HOMO SAPIENS (HUMAN) mass = 117328.1 Da, pI = 5.5

FQTTK**LNGFEVFAR**FGSAI

175.12 +/- 0.10 Da: **y"1** (175.12 Da)
334.14 +/- 0.10 Da:
387.20 +/- 0.10 Da: **a*4** (387.23 Da)
431.15 +/- 0.10 Da:
448.17 +/- 0.10 Da:
492.29 +/- 0.10 Da: **y"4** (492.29 Da)
544.24 +/- 0.10 Da: **b*5** (544.27 Da)
561.26 +/- 0.10 Da: **b5** (561.27 Da)
643.31 +/- 0.10 Da: **b*6** (643.34 Da)
660.32 +/- 0.10 Da: **b6** (660.34 Da)
825.42 +/- 0.10 Da: **y"7** (825.43 Da)
922.44 +/- 0.10 Da: **y*8** (922.47 Da)

Search time = 0 s

PROWL

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Figure 22a

PepFrag Search Results

Spectrum description:
March 8/2000 A (alpha-v) 1035.533
Database: sprot
Kingdom: primates
Maximum number of proteins in result: 10
Protein mass: 0.0-150.0 kDa
Protein pI: 0.0-15.0
Acrylamide
Enzyme: Trypsin, # of incompletes: 2

Mass of parent peptide: 1051.5 +/- 0.1 , charge state = 1+
Maximum number of phosphorylations per peptide: 0 S/T and 0 Y.
Fragments: 120.1, 175.1, 268.1, 277.1, 387.2, 415.2, 492.3, 526.2, 544.2, 615.3, 621.3, 625.3, 643.3, 768.4, 825.4, Error: 0.1, Matches: 10
Ion types: a, a*, b, b*, y", y*, Carboxypeptidase, Aminopeptidase

Searching: sprot-primates

ITAV_HUMAN VITRONECTIN RECEPTOR ALPHA SUBUNIT PRECURSOR (INTEGRIN ALPHA-V) (CD51) - HOMO SAPIENS (HUMAN) mass = 117328.1 Da, pI = 5.5

FQTTK**LNGFEVFAR**FGSAI

120.07 +/- 0.10 Da:
175.10 +/- 0.10 Da: **y"1** (175.12 Da)
268.12 +/- 0.10 Da: **b*3** (268.16 Da)
277.10 +/- 0.10 Da:
387.18 +/- 0.10 Da: **a*4** (387.23 Da)
415.18 +/- 0.10 Da: **b*4** (415.22 Da)
492.27 +/- 0.10 Da: **y"4** (492.29 Da)
526.21 +/- 0.10 Da:
544.22 +/- 0.10 Da: **b*5** (544.27 Da)
615.30 +/- 0.10 Da: **a*6** (615.34 Da)
621.31 +/- 0.10 Da: **y"5** (621.34 Da)
625.29 +/- 0.10 Da:
643.30 +/- 0.10 Da: **b*6** (643.34 Da)
768.39 +/- 0.10 Da: **y"6** (768.40 Da)
825.43 +/- 0.10 Da: **y"7** (825.43 Da)

Search time = 1 s

PROWL

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Figure 22 b

Table 1.

Peptides extracted from individual bands on the gel were analyzed through mass spectrometry. For identification or confirmation of peptide sequences, many individual peptides were further investigated using tandem mass spectrometry. For each labelled band on the gel, the peptides comprising the peptide mass fingerprint were identified and the results summarized below.

Band Identification	Peptides Characterized
A	16 alpha-v
B	18 beta-3 4 alpha-v
C	6 alpha-v 2 beta-3
D	5 alpha-v 2 beta-3
E	3 alpha-v 1 beta-3
F	3 alpha-v 1 beta-3
G	9 alpha-v 4 beta-3
H	5 alpha-v
I	2 actin 1 alpha-v
J	1 actin 1 alpha-v
K	2 alpha-v

Table 2.

Proportion of peptides from alpha-v and beta-3 with specific chemical modifications.

Modification	Alpha-v	Beta-3
No Modification	24	17
Acrylamide	11	10
Iodoacetamide	2	6
Formic Acid	15	1
Loss of NH_3	1	0
Oxidized Methionine	3	2
Total	**56**	**36**

Table 3.

Peptide recovery from macerated and non-macerated gel fragments was compared. Only the recovery of peptides from the 'alpha-v' band were compared as a preliminary test of the modification in protocol.

Number of Alpha-v Peptides Recovered from Macerated Gel Fragment (single experiment)	**Number of Alpha-v Peptides Recovered from Non-macerated Gel Fragments (4 experiments)**
22	14, 16, 17, 17

Discussion

It was the aim of this project to develop a method which could be applied to the determination of molecules associated with integrins. During affinity purification of various integrin subunits, it was observed that multiple bands appeared on the silver-stained gel which had molecular weights much lower than any integrin alpha or beta subunit. Since the conditions used for the purification were quite stringent, the presence of these lower molecular weight proteins was unexpected. We hypothesized that these lower molecular weight bands corresponded to components of supramolecular complexes which associate very strongly with the respective integrin subunit(s). To identify these unknown molecules, a reliable method for applying mass spectrometry to the characterization of these biomolecules was then developed. However, information gained from this research extends beyond the establishment of successful protocols. These experiments have brought our attention to the many potential pitfalls in both purifying proteins and obtaining (as well as analyzing) useful mass spectra.

SDS-PAGE of various purified integrin preparations revealed the presence of a number of bands other than integrin alpha and beta subunits. In an attempt to characterize these proteins, the bands were cut from the gel and individually trypsinized. The resulting peptides were extracted from each gel band and subjected to mass spectrometry and selected peptides fragmented by tandem mass spectrometry. Peptides from each fingerprint were originally selected for analysis by tandem mass spectrometry based on peak intensity. Since the fingerprint analysis by *PeptIdent* or *ProFound* had not yet been performed, there was no characterization of peptide peaks as arising from a particular protein. In subsequent experiments, the peptide peaks were selected for tandem mass spectrometry based on peak identification through

analysis of the peptide mass fingerprints. Both peptides which had been accounted for and unknown peptides were selected for fragmentation.

Advantages and Disadvantages of Mass Spectrometry:

One of the biggest advantages in using mass spectrometry is the minute amount of protein required for identification. If a protein is present even at picogram quantities, and a single peptide can be recovered from the polyacrylamide gel, sequenced, and the protein identified through this approach. Another advantage of the approach is the extremely fast generation of mass spectra from well prepared samples. The fact that it takes only moments to obtain a peptide fingerprint, and only a few more to generate a fragmentation pattern from a selected peptide, indicates that this technique has high throughput potential. The time consuming steps in the system are protein preparation and analysis of the mass spectra. As analysis programs improve and familiarity is achieved, the vast majority of time spent on these experiments will be in the protein purification and preparation. The mass accuracy (<50ppm) of the instrument allows for a high degree of confidence in the analysis of the acquired mass spectra.

There are also disadvantages to using mass spectrometry for the characterization of proteins isolated from gel bands. These disadvantages can largely be addressed through modifications in protein purification and preparation for mass spectrometry. For example, mass spectrometry is sensitive to the presence of certain detergents which increase background noise (e.g. CHAPS). To minimize background levels, these detergents need simply be avoided during the preparation procedure or eliminated in further purification steps. MALDI can be used rather than electrospray ionization (ESI) to further address this issue, as MALDI is less sensitive than ESI to detergents in general. Due to the sensitivity of the instrument, common environmental

proteins such as human keratin can be detected, which may contaminate samples in extremely low amounts. Contamination can be avoided through meticulous sample preparation and the use of non-powdered gloves at all times. Our ability to address its limitations combined with the significant advantages of mass spectrometry make this a feasible and promising approach for the characterization of integrin associated proteins. Recovery of peptides from polyacrylamide gels remains problematic. Inherent physio-chemical properties of peptides themselves may prevent efficient extraction from gel fragments. The number of steps involved in sample preparation for MALDI almost certainly results in selective loss of peptides with certain properties. Minimizing sample handling without compromising sample purity (i.e. without increasing background) may favour recovery of more peptides from any given sample. Alternatively, the use of gels which could be depolymerized may prove to enhance extraction of peptides from samples.

One disadvantage which can not be accounted for through changes in protocol is the fact that signal intensity does not correlate well with amount of sample analyzed. This reveals the difficulty in attempting to use MALDI for any type of quantitative analysis. This is highlighted by the significant differences observed when comparing mass spectra from a single sample obtained just seconds apart.

Programs used for Peptide Mass Fingerprint Analysis:

The analysis of peptide mass fingerprints were performed twice, utilizing two separate programs (*PeptIdent* and *ProFound*) in order to acquire two independent results, thus increasing confidence in the analysis. Each of the two programs has advantages over the other. For instance, *PeptIdent* will allow both carbamidomethylation (by iodoacetamide) and carbamidoethylation (by acrylamide) as potential modifications to be used simultaneously during a search of the databases.

ProFound on the other hand, will allow either one or the other of the afore mentioned carbamidomethylation /carbamidoethylation to be incorporated into a single search. However, *PeptIdent* will allow the user to search against the databases with a maximum of 2 missed cleavage sites while *ProFound* permits enzymatic cleavage to have missed more than 2 cleavage sites within a single peptide. In addition, the way in which the coverage of the protein is displayed differs between the programs. *ProFound* displays results in a cartoon type format with short horizontal lines representing the individual peptides aligned with respect to the complete amino acid sequence of the protein along the x-axis. *PeptIdent* has an alternative, and possibly more informative format for the presentation of the results. In *PeptIdent*, the entire amino acid sequence is shown and residues contained within the matched peptides are simply capitalized. This has the advantage of permitting instant analysis of regions of the molecule which are unaccounted for. In addition, this program provides the list of peptide masses found to match to a protein (same as *ProFound*), but has the added feature of displaying not only the residue numbers (as in *ProFound*), but also the amino acid sequence of these peptides. Therefore, to attain as much information as possible and limit the number of peptides left unaccounted for, it is necessary to utilize both of these programs in the analysis of peptide masses acquired from mass spectrometry.

A potential problem associated with allowing for multiple chemical modifications in one fingerprint analysis is an increased possibility of obtaining false positives. This occurs simply as a result of increasing the number of potential peptide combinations that could be matched to the database. Another program called *FindMod* was utilized to aid in the identification of peptides not found to match the alpha-v or beta-3 peptide fingerprint (data not shown). The accession number of the protein suspected to give rise to the peptides is entered into the program along with any of the peptide masses that are in question. *FindMod* then analyzes the data and

returns a list of peptides from the peptide fingerprint of the suspected protein and the chemical modifications which could have given rise to the unidentified peptides. The suggested parent peptide mass is then used to analyze the peptide fragmentation pattern generated from these peptides. As the correct parent peptide mass is critical for proper identification of the amino acid sequence from its peptide fragmentation pattern, the identification of the potential chemical modification is a critical step in the analysis of peptide fingerprints. Therefore, there is an intricate exchange of information necessary between peptide fingerprinting and peptide fragmentation patterns (MS/MS) in order to successfully identify every peptide within an individual fingerprint

An additional consideration in using the current software for the analysis of acquired peptide fingerprints or fragmentation patterns is that the software which is presently available is lagging slightly behind the technical advances being made in the area. For instance, details on how *PeptIdent* and *ProFound* differ with respect to data entry, modifiable parameters, and output format make it almost mandatory to perform the analysis using each program independently and compare the obtained results. However, even in utilizing both of these programs, many potential chemical and post-translational modifications are unaccounted for, leaving unmatched peptides to be further analyzed using yet another program or programs (e.g. *FindMod*). The absence of a comprehensive program which takes into account all of the potential modifications and for inclusion of the complete experimental variables makes the analysis of mass spectrum much more cumbersome than theoretical analysis suggests. For example, post-translational modifications may not be incorporated into the analysis of fingerprint analysis using either *ProFound* or *PeptIdent*, leaving the researcher looking to other programs to identify potential post-translational or chemical modifications which could be responsible for the mass difference. However, in order to utilize a program which can predict modifications of a peptide, the protein

from which the peptide came must be known. The reason for this is that without this input, a program like *FindMod* would have to search the entire database looking for potential modifications of every peptide which could possibly result in the mass of the peptide in question. Even if the program would perform such an analysis, the output file would contains so many candidates, it would be enormous as well as uninformative. This necessitates identification of the protein(s) in the sample through peptide mass fingerprint or MS/MS analysis. Following this preliminary analysis, the identity of the protein can be used as an input parameter. The program then uses actual peptide masses from the identified molecule to suggest modifications that could have given rise to the masses of the unmatched peptides. This technique can be used in combination with tandem mass spectrometry to provide a clear identification of the peptide in question.

Programs used for Peptide Fragmentation Pattern Analysis:

MsTag and PepFrag each provide unique options for the analysis of fragmentation patterns. For instance, *PepFrag* allows for the presence of phosphorylated residues within a peptide while MsTag lacks this capability. On the other hand, *MsTag* (but not *PepFrag*) is capable of recognizing and matching internal ions from the fragmentation pattern. It is therefore suggested that both programs be utilized to maximize the number of ions which can be identified, as certain ions will only be recognized by one or the other. Regardless of the program used for the analysis, the proper mass of the parent peptide is essential. In the case of a modified peptide, the mass of the parent ion would be altered and therefore prevent these programs from assigning the true amino acid sequence to the peptide. However, *FindMod* can be used to suggest potential chemical modifications to parent peptides, and these unmodified peptide masses can be tested through *MsTag* or *PepFrag*. The

FindMod program is useful in that it allows the user to enter unmatched peptide masses along with the suspected parent protein in an attempt to characterize potential modifications. The program essentially predicts modifications of peptides from the parent protein which could have given rise to the unmatched peptide masses. *FindMod* can be manipulated to allow for phosphorylation, all known splice variants of a protein, missed cleavages, glycosylation, single amino acid substitutions, and many other potential chemical and post-translational modifications which are absent from *PeptIdent* and *ProFound.*

To understand why *MsTag* and *PepFrag* would be incapable of assigning a sequence to the peptide, one must first understand how these programs actually 'sequence' the peptide from its respective fragmentation pattern. The fact is that these programs don't actually sequence the peptide. Rather, they search the designated database for peptides of the appropriate mass which meet the conditions specified (e.g. enzyme used, missed cleavages allowed). Once the peptides of appropriate mass have been identified, the program obtains 'virtual' or predicted fragmentation patterns, which are based on the unique amino acid sequences of each individual peptide. The experimentally acquired fragmentation from the peptide in question is then compared to the 'virtual' fragmentation patterns. Matching the daughter ions from the actual fragmentation pattern to a 'virtual' fragmentation pattern signifies the amino acid sequences are identical. This is the indirect way in which a peptide fragmentation pattern is assigned an amino acid sequence and illustrates the requirement for the correct peptide mass (without any modifications) to be entered as input.

Choosing a Database to Search:

In selecting the database(s) to be searched by the various peptide fingerprint analysis programs, NCBI-nr and SwissProt were chosen. NCBI-nr is a database which

contains amino acid sequences for known proteins as well as predicted amino acid sequences for incompletely characterized stretches of DNA. The SwissProt database contains records for only those proteins that have been highly annotated and contain information regarding chromosomal location, functional significance, homologous proteins, and other characteristics. This database when used in conjunction with TrEMBL would yield the same results as if NCBI-nr was searched. That is to say that TrEMBL contains records for incompletely characterized proteins and nucleotide sequences, and that TrEMBL + SwissProt = NCBI-nr. In general, both NCBI and SwissProt were utilized independently to verify results obtained from the other. It should be noted that this approach to confirmation could only be used if the protein of interest has been well characterized. In the case of this study, the only proteins identified were alpha-v or beta-3 integrin subunits, and actin, all of which are highly annotated proteins that can be found in SwissProt. If however there were unknown or poorly characterized proteins in a given sample, this method of confirmation would not apply. In general, the NCBI-nr database should be used initially, and more specialized databases could be searched subsequently depending on the search results obtained.

It should also be noted that in using peptide fingerprint or peptide fragmentation pattern analysis programs, the user must carefully select the appropriate phylogenetic category. For instance, searching all eukaryotic records instead of the specific genus from which the protein was isolated, may yield large numbers of false positive matches. While searching expressed sequence tag (EST) databases may occassionally provide useful information, the current view of EST databases is rapidly changing and their significance is in question. This type of approach is also significantly more time consuming and yields data sets which are extremely complicated to navigate and interpret. By selectively narrowing your search criteria,

the processing time will be decreased while the obtained results will be more easily managed and interpreted.

Peptide mass fingerprint analysis of affinity purified Alpha-v/Beta-3:

Prior to performing mass spectrometry on the proteins within the silver-stained gel, it was believed that the bands at ~130kDa and ~115kDa corresponded to alpha-v and beta-3 integrin subunits respectively. This assumption was made based on the known molecular weights for these subunits as well as the relative intensity of the bands themselves. Since the affinity purification procedure was directed at the integrin alpha-v/beta-3 heterodimer, the two bands of highest intensity may then be expected to correspond to the individual subunits comprising the intact integrin. Analysis of the acquired mass spectra from these alpha-v/beta-3 integrin preparations suggested the presence of the alpha-v as well as beta-3 subunit peptides in multiple bands on the gel. Peptide mass fingerprint analysis of the intense band at ~130kDa revealed a large number of chemically unmodified peptides which were easily identifiable as arising from the tryptic digest of the alpha-v integrin subunit. Incorporating potential chemical modifications into the fingerprint analysis of this band revealed another subset of chemically altered peptides belonging to the alpha-v integrin subunit. Analysis of the band at ~115kDa revealed the second component of the purified heterodimer, the beta-3 subunit. Modifications during sample preparation such as addition of acrylamide adducts of cysteine residues, carbamidomethylation of cysteines by iodoacetamide, and oxidation of methionines can be included in the analysis of the fingerprint using *PeptIdent* and *ProFound*. Allowing for these chemically altered peptides greatly increased the number of peptides matching the integrin alpha-v subunit tryptic fingerprint. Still other modifications to the peptides are possible and yet can not be accounted for during *PeptIdent* or *ProFound* anlaysis

of the obtained peptide mass fingerprints. These chemical modifications, such as methylation by formic acid, can be included in the analysis only if manually calculated.

The number of peptides recovered from a gel band in an individual experiment was always less than the total recovered peptides from all extractions of the same band. In the case of the 'alpha-v' (130kDa) band, 40 peptides were recovered overall, corresponding to approximately 43% coverage of the protein. The highest recovery from a single experiment was 22 peptides spanning 27% of the molecule. As for the 'beta-3' (115kDa) band, a total of 34 peptides were recovered overall, accounting for nearly 39% of the protein. The recovery for a single experiment was again similar to the results obtained for alpha-v. As not all peptides corresponding to alpha-v or beta-3 were recovered from the polyacrylamide gel, the underlying mechanism responsible for differential recovery of peptides between experiments is unclear at this point. Differences in physio-chemical properties of individual peptides will account for variations in solubility, charge, hydrophobicity, and many other criteria influencing recovery at multiple stages during processing.

Identification of Integrin Peptides in Lower Molecular Weight Bands:

Both alpha-v and beta-3 peptides were recovered and identified from each and every band on the gel. The presence of alpha-v in multiple bands on the gel was later confirmed by western blot of the same preparations (data not shown). It is believed that endogenous proteases from the placenta degraded the integrin subunits during the purification process. Some of this degraded integrin is likely to arise from digestion of immature integrin, as they lack glycosylation found decorating the mature integrin subunits. Glycosylation of the mature integrin molecule may protect the molecule

from certain types of endogenous enzymatic digestion during the initial emulsification and extraction procedures.

The fact that so many alpha-v and beta-3 breakdown products were found suggests several things. In looking at the silver-stained gel of the alpha-v/beta-3 preparation, it would seem that quite a few bands other than the alpha-v and beta-3 are present. Prior to analysis by mass spectrometry, this was originally interpreted as a plethora of associated proteins. Without the use of mass spectrometry, it is not likely that these 'associated proteins' would have been identified (at least not in the time frame of this master's thesis). Therefore, we have saved valuable time and effort from being wasted screening antibodies directed against each individual potentially associated protein. The finding that each of the bands corresponds to a breakdown product of alpha-v, beta-3, or both, informs us that the conditions used for affinity purification are not acceptable with respect to protease inhibition. Further purifications will have to incorporate a much more comprehensive protease inhibitor cocktail in order to prevent this degradation of intact integrin from occurring. If we were unsuccessful in preventing digestion of the covalent linkages of peptide bonds, there is little mystery as to why we were unable to preserve the much weaker non-covalent interactions necessary for co-precipitation of associated molecules.

The antibody, LM-609, which was used for the affinity column during the purification of the alpha-v/beta-3 integrin has been demonstrated to recognize a conformational epitope dependent on the association of alpha-v and beta-3. It can therefore be determined that the integrin heterodimer was isolated as such, and not as individual subunits. Furthermore, this property of LM-609 to recognize alpha-v/beta-3 heterodimers permits the assumption that each of the breakdown products contains at least a portion of the epitope required for recognition by the antibody and that these fragments retained the association with the complementary subunit (at least until gel separation).

Actin Peptides:

The recovery and identification of actin peptides from the alpha-v/beta-3 preparation was originally promising, suggesting the presence of another associated protein seemingly required to provide the integrin-actin linkage. However, after further analysis, no such cytoskeletal element (e.g. talin, alpha-actinin) could be identified in the preparation. This led to the conclusion that either actin was a contaminant (much the same as described for keratin), or that actin could bind to the alpha-v/beta-3 dimer directly. After investigating each of these possibilities, there are no reports incriminating actin as a contaminant, or as having a direct association with any integrin subunit. It is our conclusion that the actin in this case is present as a contaminant and does not directly associate with integrin. Identifying actin as a contaminant is not so unusual when taken into account the fact that actin comprises approximately 10% of the total protein in any given cell. Its abundance combined with the extremely high sensitivity of mass spectrometry could account for the identification of actin peptides in these samples.

Fragmentation Pattern Analysis:

The fragmentation patterns obtained through tandem mass spectrometry were also analyzed twice, again using two separate programs (*MsTag* and *PepFrag*) in order to increase our confidence in the results obtained. All of the peptides investigated through tandem mass spectrometry were found to correspond excellently with the predicted amino acid sequences of the respective peptides from the original fingerprints. While the programs quite consistently give the same results when the same information and parameters are entered, one or the other program occasionally identifies matches (from the fragmentation pattern) that the other does not. (Compare

Figure 18 and 19 for an example). Again, each of these programs has certain advantages over the other, and each researcher must determine which is best suited to analysis of the data acquired from their experiments. Utilizing both takes advantage of the strengths of each of the programs while masking the potential weaknesses that could be realized through the use of either one.

Improving Quality of Peptide Fingerprints:

Over the course of the many experiments it was observed that peptides corresponding to trypsin were being generated at high levels, and were perhaps compromising the signal from sample peptides. Peptide peaks of high intensity cause remaining peptide peaks to appear quite small and potentially insignificant when compared to background noise. To address this issue, the concentration of trypsin used for the overnight in-gel digestion was investigated. A compromise must be struck whereby the sample proteins are efficiently digested and the peptides generated from trypsin itself are kept to a minimum. It was found that dropping the concentration of trypsin from 12ug/ml to 5ug/ml was the effective for both concerns. This was the concentration of trypsin which best suited the amounts of protein within the gel bands. For lighter bands (containing less protein) it would follow then that the concentration may need to be dropped even further to again strike the correct balance. For this series of experiments, using 2.5ug/ml of trypsin was found to be ineffective for digestion of the sample proteins. Under these conditions, uncleaved peptides may not be efficiently extracted from gel fragments and/or may be too large to be within the useful m/z range. It should also be noted that increasing the amount of protein loaded onto the gel does not necessarily improve the quality of the corresponding peptide fingerprint. Again, this depends to some extent on the ratio of trypsin to

sample protein, and must be taken into consideration when increasing (or decreasing) the amount of protein loaded onto the gel.

Improving Recovery of Peptides from Gel Fragments:

Upon finding that certain protocols incorporated a step for maceration of gel pieces, an experiment was conducted to investigate any potential increases in peptide recovery due to such treatment. The results indicated that an increased number of peptides are recovered from macerated gel pieces when compared to intact gel pieces containing the same protein (from the same preparation). Since the recovery of peptides from the gel was increased, the coverage of the protein was also improved. It is therefore suggested that maceration be incorporated into the protocol in order to maximize the percentage of protein accounted for by the recovered peptides. The fact that peptides covering 100% of the intact protein could not be recovered may suggest that some peptides are broken down (during trypsinization) to peptide fragments that are too small to contribute in any useful manner to the fingerprint. Alternatively, peptides spanning more than approximately 30 amino acids without cleavage at an arginine or lysine residue may not be visualized in the m/z range used (approx. 500-3500) and may be too large to contribute to the fingerprint with any merit. Essentially, the longer/larger the peptide, the greater the number of potential amino acid combinations that could comprise such a peptide. The smaller the peptide, the greater the chance that the amino acid sequence comprising the peptide will appear randomly in proteins within the database searched. To further complicate the issue, it appears as though some peptides which we would expect to recover remain 'stuck' in the polyacrylamide gel. These peptides may be differentially soluble in solvents alternative to formic acid and TFA.

Reducing Chemical Modifications of Recovered Peptides:

As analysis of peptide mass fingerprints can be complicated, and thus quite time consuming, an effort was made to reduce potential chemical modifications. An experiment was performed to examine the possible prevention of formic acid modification, by simply substituting trifluoroacetic acid for formic acid at the stage of peptide extraction from the gel pieces. The use of 0.1% TFA in place of 5% formic acid resulted in the absence of the +28 and +56 forms of the parent peaks. This served two purposes: (1) to reduce the number of peaks in the peptide mass fingerprint which could not be accounted for by any current fingerprint analysis program, and (2) to prevent the associated reduction in intensity of the unmodified parent peak. The resulting peptide mass fingerprint appeared much less complicated with reference to the number of the total number of peaks. However, it should be noted that the maximal coverage of total protein sequence recovered in the form of peptides from a gel piece occurred in an experiment using the extraction procedure which included the formic acid. This may relate to the relative solubility differences between formic acid and TFA.

Considerations for Future Experiments:

Preparation of the integrin samples may prove to be problematic for investigating potential associations as the stringent purification conditions may not allow for weak interactions of proteins with the integrin. This may prove to be extremely important as many of the integrin associations are thought to be transient and therefore may not be preserved during the integrin purification procedure. Through the use of milder detergents and less stringent conditions in general, it should be much easier to maintain the non-covalent (and likely weak) associations with

purified integrins. It appears obvious that a more comprehensive cocktail of protease inhibitors is necessary to prevent the desired intact proteins from being degraded during the purification procedure. Once these details have been worked out, the application of this approach is virtually unlimited. For instance, a comparison can be made between integrin associated proteins isolated from activated vs. unstimulated (and normally non-adherent) cell lines. If activation alters the adhesive properties of the cell line, the differences between the two profiles could provide insight into the mechanism of integrin activation as well as cellular adhesion and migration. Furthermore, the use of cell lines for elucidation of potential associations may ultimately prove more useful than the use of integrins purified from tissue (such as placenta). The reason for this is quite simply that a cell line represents a clonotypic cellular population while tissue homogenization will potentially contain dozens of cell types. For this reason, the analysis of integrin associated proteins purified from tissue would likely be extremely complicated. Since integrin can be purified in large quantities from placenta, this is the initial source we chose to establish the protocol and techniques required for further experiments involving cell lines and ultimately peripheral blood cells. The information acquired from the identification of integrin associations in various cell lines could be used to aid in predicting the types of supramolecular complexes we may observe in further studies involving physiologically relevant peripheral blood cells.

Following the identification of molecules involved in integrin-containing complexes, the presence of such proteins could then be visualized through the combined use of gel electrophoresis and western blotting with antibodies directed against identified components. Co-precipitation experiments could also be utilized for the immunoprecipitation of the identified protein in order to show integrin can be co-purified with the candidate molecule. This reciprocal co-purification technique leaves little doubt with respect to the proposed interaction as there is almost no chance of

cross-reactivity occurring in both immunoprecipitations reactions. Immunofluorescence microscopy experiments could confirm the co-localization of various components, while functional studies could then be used to show that the association is of biological significance. Blocking antibodies directed against the candidate molecule on intact cells would reveal the influence of a molecule on integrin-dependent cellular adhesion and/or migration. Transfection of a cell line with a fusion protein (comprised of the cytoplasmic domain of the candidate molecule and the extracellular domain of an irrelevant molecule) could be used in order to gain an understanding of contribution the candidate molecule makes to integrin function. For example, is the association important for signaling events or does the molecule play more of a role as an adaptor protein? Another question that could be addressed by these transfection studies is whether the cytoplasmic, transmembrane, or extracellular domain is responsible for the suggested interaction with integrin.

Future experiments utilizing less stringent conditions for integrin affinity purification will undoubtedly lead to the identification of many proteins associated with the respective integrins. One potential association which has not yet been discussed is integrin interaction with transmembrane matrix-metalloproteinases. Recent unpublished work by Strongin et al has described a functional relationship between alpha-v/beta-3 integrin and membrane-type matrix-metalloproteinase (MT-MMP). Co-precipitation experiments showed association of these two proteins, while antibody blocking and single and double transfection experiments were used to investigate the potential functional relationship. It was shown that the association leads to modification of the beta-3 integrin subunit which results in a negative mobility shift of 5kDa. In addition, the presence of the MT-MMP greatly increased cellular migration on vitronectin and the conversion of another matrix-metalloproteinase from an inactive pro-enzyme to a mature gelatinolytic enzyme. This functional relationship appears to shed some light on the increased migratory

behaviour of metastatic cancers. The integrin alpha-v/beta-3 used in these experiment has been purified from placenta, which is another rapidly growing tissue capable of mediating angiogenesis. The described association of this integrin with MT-MMPs may explain the unique ability of placenta to mediate the angiogenic process required for establishment and maintenance of the mature tissue. Although only one intense band contains significant beta-3 peptides in our hands, other bands of lower intensity have been shown to contain a few such peptides. Whether or not one of these lower bands represents the MT-MMP-modified form of the beta-3 integrin is not clear. Further investigation of the associated proteins is required to whether MT-MMPs are even associated with alpha-v/beta-3 in placental tissue.

Future Epitope Mapping Experiments:

This approach should also prove to be useful in epitope mapping, where monoclonal antibodies (of unknown epitope recognition) can be covalently coupled to beads and used to affinity purify the respective antigen/protein. Limited proteolysis can then be used to digest away the majority of the protein without digestion of the monoclonal antibody (mAb) as glycosylation at strategic sites on the immunoglobulin molecule will protect it from being digested. Elution of the remaining peptide (containing the epitope) followed by identification by mass spectrometry can provide information regarding the amino acid sequence recognized by the antibody. If further characterization is required, endopeptidases can then be used with controlled incubation times to sequentially remove single amino acids from either the carboxy- or amino-termini. This process of removing single amino acids should continue until the enzyme reaches an amino acid protected from digestion due to its association with (or binding in) the peptide binding groove of the antibody. The mechanism is not unlike

the Edman degradation, and can be used to rapidly characterize the specific epitopes recognized by a number of mAbs from this lab such as N29, B44, and 3S3.

Conclusions:

To conclude then, the main objectives of this research project were three-fold. The first objective was to establish a method for identifying proteins through the application of mass spectrometry. The second goal was to apply this procedure to affinity purified integrin preparations. Both of these objectives were indeed fulfilled as methods were established which allowed the identification of alpha-v and beta-3 integrin subunits from several integrin preparations. The third objective of characterizing proteins which co-purify with various integrin heterodimers was indirectly achieved. Under the stringent conditions of integrin purification, no associated protein interactions with alpha-v/beta-3 were maintained. However, given the fact that the vast majority of peptides analyzed from each band on the gel could be identified using this approach, it follows then that had any interactions been retained, these molecules would have been characterized. Actin, while present as a contaminant, was identified through tandem mass spectrometry of two individual peptides from one band on the gel. This sensitivity affords the confidence that associated proteins will be identified under conditions permitting molecular associations.

References

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

Albiges-Rizo C, Frachet P, and Block MR. Down regulation of talin alters cell adhesion and the processing of the alpha-5/beta-1 integrin. J. Cell Sci. 1995; 108: 3317-3329.

Balzac F, Belkin AM, Koteliansky VE, Balabanov YV, Altruda F, Silengo L, and Tarone G. Expression and functional analysis of a cytoplasmic domain variant of the beta-1 integrin subunit. J. Cell Biol. 1993; 121: 171-178.

Balzac F, Retta SF, Albini A, Melchiorri A, Koteliansky VE, Geuna M, Silengo L, and Tarone G. Expression of beta-1B integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. J. Cell Biol. 1994; 127: 557-565.

Belkin AM, Zhidkova NI, Balzac F, Altruda F, Tomatis D, Maier A, Tarone G, Koteliansky VE, and Burridge K. Beta-1D integrin displaces the beta-1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. J. Cell Biol. 1996; 132: 211-226.

Behr S and Schriever F. Engaging CD19 or target of an antiproliferative antibody 1 on human B lymphocytes induces binding of B cells to the interfollicular stroma of human tonsils via integrin alpha-4/beta-1 and fibronectin. J. Exp. Med. 1995; 182: 1191-1199.

Berditchevski F, Bazzoni G, and Hemler ME. Specific association of CD63 with the VLA-3 and VLA-6 integrins. J. Biol. Chem. 1995; 30: 17784-17790.

Bretscher MS. Circulating integrins: alpha-5/beta-1, alpha-6/beta-4 and Mac-1, but not alpha-3/beta-1, alpha4/beta-1 or LFA-1. EMBO J. 1992; 11: 405-410.

Brindle NPJ, Holt MR, Davies JE, Price CJ, and Critchley DR. The focal-adhesion vasodilator-stimulated phosphoprotein (VASP) binds to the proline-rich domain in vinculin. Biochem. J. 1996; 308: 753-757.

Burlingame AL, Boyd RK, and Gaskell SJ. Mass spectrometry. Anal. Chem. 1994; 66: 634R-683R.

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

Butcher EC. Leukocyte-endothelial cell recognition: three steps to specificity and diversity. Cell. 1991; 67: 1033-1036.

Calderwood DA, Zent R, Grant R, Rees DJG, Hynes RO, and Ginsberg MH. J. Biol. Chem. 1999; 274 (40): 2071-28074.

Cary LA, Han DC, and Guan J-L. Integrin-mediated signal transduction pathways. Histol. Histopathol. 1999; 14: 1001-1009.

Chen HC, Appeddu PA, Isoda H, and Guan JL. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J. Biol. Chem. 1996; 271: 26329-26334.

Choquet D, Felsenfeld DP, and Sheetz MP. Ectracellular matrix rigidity causes strenthening of integrin-cytoskeleton linkages. Cell. 1997; 88: 39-48.

Clark EA and Brugge JS. Integrins and signal transduction pathways: the road taken. Science. 1995; 268: 233-239.

Crawford AW, Michelson JW, and Beckerle MC. An interaction between zyxin and alpha-actinin. J. Cell Biol. 1992; 116: 1391-1393.

Critchley DR, Holt MR, Barry ST, Priddle H, Hemmings L, and Norman J. Biochem. Soc. Symp. 1998; 65: 79-99.

D'Souza SE, Ginsberg MH, Lam SC-T, and Plow E. Chemical cross-linking of arginyl-glycyl-aspartic acid peptides on adhesion receptors on platelets. Science. 1988; 242: 91-93.

Dedhar S. Integrins and signal transduction. Curr. Opin. Hematol. 1999; 6: 37-43.

Dedhar S, Williams B, and Hannigan G. Interin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Cell. Biol. 1999; 9: 319-323.

Dehdar SD and Hannigan GE. Integrin cytoplasmic interactions and bi-directional transmembrane signaling. Curr. Opin. Cell. Biol. 1996; 8: 657-669.

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J and Dedhar S. Phosphoinositide-2-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. USA. 1998; 95: 11211-11216.

Deryugina EI, Bourdon MA, Jungwirth K, Smith JW, and Strongin AY. Functional activation of integrin alpha-v/beta-3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int. J. Cancer. 2000; 86: 15-23.

Drickamer K. Two distinct classes of carbohydrate-recognition domains in animal lectins. J. Biol. Chem. 1988; 263: 9557-9560.

Ebnet K and Vestweber D. Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. Histochem. Cell. Biol. 1999; 112: 1-23.

Fabbri M, Bianchi E, Fumagalli L, and Pardi R. Regulation of lymphocyte traffic by adhesion molecules. Inflamm. Res. 1999; 48: 239-246.

Fitter S, Tetaz TJ, Merndt MC and Ashman LK. Molecular cloning of cDNA encoding a novel platelet-endothelial cell tetra-span antigen, PETA-3. Blood. 1995; 86: 1348-1355.

Gilmore AP, Jackson P, Waites GT, and Critchley DR. Further characterisation of the talin-binding site in the cytoskeletal protein vinculin. J. Cell Sci. 1992; 103: 719-731.

Gumbiner BM. Proteins associated with the cytoplasmic surface of adhesion molecules. Neuron. 1993; 11: 551-564.

Guan J-L, Trevithick JE, and Hynes RO. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul. 1991; 2: 951-964.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC and Dedhar S. Regulation of cell adhesion and anchorage-dependent growth by a new 1-integrin-linked protein kinase. Nature. 1996; 379: 91-96.

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

Hemler ME, Mannion BA, and Berditchevski F. Association of TM4SF proteins with integrins: relevance to cancer. Biochim. Biophys. Acta. 1996; 1287: 67-71.

Hemmings L, Rees DJ, Ohanian V, Bolton SJ, Gilmore AP, Patel B, Priddle H, Trevithick JE, Hynes RO, and Critchley DR. Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J. Cell Sci. 1996; 109: 2715-2726.

Henzel WJ, Billeci TM, Stults JT, and Wong SC. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc. Natl. Acad. Sci. U.S.A. 1993; 90: 5011-5015.

Horwitz A, Duggan K, Buck C, Beckerle MC, and Burridge K. Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. Nature (London). 1986; 320: 531-533.

Huang Y. and Wu C. Integrin-linked kinase and associated proteins (Review). Int. J. Mol. Med. 1999; 3: 563-572.

Hunkapiller M, Kent S, Caruthers M, Dreyer W, Firca J, Giffin C, Horvath S, Hunkapiller T, Tempst P, and Hood L. A microchemical facility for the analysis and synthesis of genes and proteins. Nature. 1984; 310: 105-111.

Huttlemaier S, Bubeck P, Rudiger M, and Jockusch BM. Characterization of two F-actin-binding and oligomerization sites in the cell-contact protein vinculin. Eur. J. Biochem. 1997; 247: 1136-1142.

Hyatt SL, Liao L, Chapline C, and Jaken S. Correlation between protein kinase C binding proteins and substrates in REF52 cells. Biochemistry. 1994; 33: 1223-1228.

Hynes RO. Integrins, a family of cell surface receptors. Cell. 1987; 48: 549-555.

Jenkins AL, Nannizzi-Alaimo L, Silver D, Sellers JR, Ginsberg MH, Law DA, and Phillips DR. Tyrosine phosphorylation of the beta-3 cytoplasmic domain mediates integrin-cytoskeletal interactions. J. Biol. Chem. 1998; 273: 13878-13885.

Johnson RP and Craig SW. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. Nature. 1995; 373: 261-264.

Kang F, Laine RO, Bubb MR, Southwick FS, and Purich DL. Profilin interacts with the Gly-Pro-Pro-Pro-Pro-Pro sequences of vasodilator-stimulated phosphoprotein (VASP): implications for actin-based Listeria motility. Biochemistry. 1997; 36: 8384-8392.

King WG, Mattaliano MD, Chan TO, Tsichlis PN and Brugge JS. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol. Cell. Biol. 1997; 17: 4406-4418.

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

Kornberg L, Earp HS, Turner CE, Procktop C, and Juliano RL. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta-1 integrins. Proc. Natl. Acad. Sci. USA. 1991; 88: 8392-8396.

Kroemker M, Rudiger AH, Jockusch BM, and Rudiger M. Intramolecular interactions in vinculin control alpha-actinin binding to the vinculin head. FEBS Lett. 1994; 355: 259-262.

Lawson MA and Maxfield FR. Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. Nature (London). 1995; 377: 75-79.

Lo SH and Chen LB. Focal adhesion as a signal transduction organelle. Cancer Metastasis Rev. 1994; 13: 9-24.

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

Maecker HT, Todd SC, and Levy S. The tetraspanin superfamily: molecular facilitators. FASEB. 1997; 11: 429-442.

Mann M, Hojrup P, and Roepstorff P. Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biol. Mass Spectrom. 1993; 22: 338-345.

Mannion BA, Berditchevski F, Kraeft S-K,, Chen LB, and Hemler ME. J. Immunol. 1996; 157: 2039-2047.

Marcantonio EE and Hynes R. Antibodies to the conserved cytoplasmic domain of the integrin beta-1 subunit react with proteins in vertebrates, invertebrates, and fungi. J. Cell Biol. 1988; 106: 1765-1772.

Marsden M and Burke RD. Cloning and characterization of novel beta integrin subunits from a sea urchin. Dev. Biol. 1997; 181: 234-245.

McGregor A, Blanchard AD, Rowe AJ, and Critchley DR. Identification of the vinculin-binding site in the cytoskeletal protein alpha-actinin. Biochem. J. 1994; 301: 225-233.

Nakamura K, Iwamoto R, and Mekada E. Membrane anchored heparin-binding EGF-like growth factor (HB-EGF) and diptheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha-3/beta-1 at cell-cell contact sites. J. Cell Biol. 1995; 129: 1691-1705.

Nermut MV, Green NM, Eason P, Yamada SS, and Yamada KM. Electron microscopy and structural model of human fibronectin receptor. EMBO J. 1988; 7: 4093-4099.

Nishizaka T, Shi Q, and Sheetz MP. Postition-dependent linkages of fibronectin-integrin-cytoskeleton. Proc. Nat. Acad. Sci. USA. 2000; 97: 692-697.

Nuckolls GH, Romer LH, and Burridge K. Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts. J. Cell Sci. 102: 753-762.

Otey C, Vasquez G, Burridge K, and Erickson B. Mapping of the alpha-actinin binding site within the beta-1 integrin cytoplasmic domain. J. Biol. Chem. 1993; 268: 21193-21197.

Palacek SP, Huttenlocher A, Horwitz AF, and Lauffenburger DA. Physical and biochemical regulation of integrin release during rear detachment of migrating cells. J. Cell Sci. 1998; 111: 929-940.

Povalko FM and LaRoche SM. Activation of human neutrophils induces an interaction between the integrin beta2-subunit (CD18) and the actin binding protein alpha-actinin. J. Immunol. 1993; 151: 3795-3807.

Plaff M, Liu S, Erie DJ, and Ginsberg MH. Integrin beta cytoplasmic domains differentially bind to cytoskeletal proteins. J. Biol. Chem. 1998; 273: 6104-6109.

Radeva G, Petrocelli T, Behrend E, CHungyee L-H, Filmus J, Slingerland J and Dedhar S. Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. J. Biol. Chem. 1997; 272: 13937-13944.

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

Rees DJ, Ades SE, Singer SJ, and Hynes RO. Sequence and domain structure of talin. Nature. 1990; 347: 685-689.

Regen CM and Horwitz AF. Dynamics of beta-1 integrin-mediated adhesive contacts in motile fibroblasts. J. Cell. Biol. 1992; 119: 1347-1359.

Reinhard M, Rudiger M, Jockusch BM, and Walter U. VASP interaction with vinculin: a recurring theme of interactions with prolin-rich motifs. FEBS Lett. 1996; 399: 103-107.

Reszka AA, Hayashi Y, and Horwitz AF. Identification of amino acid sequences in the integrin beta-1 cytoplasmic domain implicated in cytoskeleton association. J. Cell Biol. 1992; 117:1321-1330.

Rodriguez Fernandez JL, Geiger M, Salomon D, and Ben-Ze'ev A. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. J. Cell Biol. 1993; 122: 1285-1294.

Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, and Chait BT. The yeast nuclear complex: composition, architecture, and transport mechanism. J. Cell. Biol. 2000; 148: 635-651.

Rubinstein E, Le Naour F, Lagaudriere-Gesbert C, Billiard M, Conjeaud H, and Boucheix C. CD9, CD63, CD81, and CD82 are components of a surface tetraspan

network connected to HLA-DR and VLA integrins. Eur. J. Immunol. 1996; 26:2657-2665.

Sampath R, Gallagher PJ, and Pavalko FM. Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associateons with talin and alpha-actinin. J. Biol. Chem. 1998; 273: 33588-33594.

Sancho D, Yanez-Mo M, Tejedor R, Sanchez-Madrid F. Activation of peripheral blood T cells by interaction and migration through endothelium: role of lymphocyte function antigen-1/intercellular adhesion molecule-1 and interleukin-15. Blood. 1999; 93: 886-896.

Schaller MD, Hildebrand JD, Shannon JD, Fox JX, Vines RR, and Parsons JT. Autophosphorylation of the focal adhesion kinase, $pp125^{FAK}$, directs SH2-dependent binding of $pp60_{src}$. Mol. Cell. Biol. 1994; 14: 1680-1688.

Schevchenko A, Wilm M, Vorm O, and Mann M. Mass spectormetric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 1996; 68: 850-858.

Sharma CP, Ezzell RM, and Arnaout MA. Direction interaction of filamin (ABP-280) with the beta-2 integrin subunit CD-18. J. Immunol. 1995; 154: 3461-3470.

Shaw AR, Domanska A, Mak A, Bilchrist A, Dobler K, Visser L, Poppema S, Fliegel L, Letarte M, and Willett BJ. Ectopic expression of human and feline CD9 in a human B cell line confers beta-1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. J. Biol. Chem. 1995; 270: 24092-24099.

Sheetz Mp, Felsenfeld DP, and Galbraith CG. Cell migration: regulation of force on extracellular-matrix-integrin complexes. Trends Cell Biol. 1998; 8: 51-54.

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

Simons K, and Ikonen E. Functional rafts in cell membranes. Nature. 1997; 387: 569-572.

Shibasaki F, Fukami K, Fukui Y, and Takenawa T. Phosphatidylinositol 3-kinase binds to alpha-actinin through the p85 subunit. Biochem. J. 1994; 302: 551-557.

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

Slupsky, JR, Seehafer JG, Tang SC, Masellis-Smith A, and Shaw AR. Evidence that monoclonal antibodies against CD9 antigen induce specific association between CD9

and the platelet glycoprotein IIb-IIIa complex. J. Biol. Chem. 1989; 264: 12289-12293.

Skubitz KM, Campbell KD, Iida J, and Skubitz APN. CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. J. Immunol. 1996; 157: 3617-3626.

Smilenov LB, Mikhailov A, Pelham RJ Jr, Marcantonio EE, and Gundersen GG. Focal adhsion motility revealed in stationary fibroblasts. Science. 1999; 286: 1172-1174.

Smith JW and Cheresh SA. The Arg-Gly-Asp-binding domain of the vitronectin receptor. J. Biol. Chem. 1988; 263: 18726-18731.

Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multi-step paradigm. Cell. 1994; 76: 301-314.

Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science. 1991; 251: 1451-1455.

Tapley P, Horwitz A, Buck C, Duggan K, and Rohrschneider L. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. Oncogene 4. 1989; 325-353.

Todd SC, Lipps SG, Crisa L, Salomon DR and Tsoukas CD. CD81 expressed on human thymocytes mediates integrin activation and IL-2-dependent proliferation. J. Exp. Med. 1996; 184: 2055-2060.

Tu Y, Li F, Goicoechea S and Wu C. The LIM-only protein PINCH directly interacts with the integrin-linked kinase (ILK) and is recruited to integrin-rich sites in spreading cells. Mol. Cell. Biol. 1999; 19: 2425-2434.

Turner CE, Glenney Jr. J, and Burridge K. Paxillin: a new vinculin-binding protein present in focal adhesions. J. Cell Biol. 1990; 111: 1059-1068.

Vicente-Manzanares M, Montoya MC, Mellado M, Frade JM, del Pozo MA, Nieto M, de Landazuri MO, Martinez-A C, and Sanchez-Madrid F. Teh chemokine SDF-1 alpha triggers a chemotactic response an d induces cell polarization in human B lymphocytes. Eur. J. Immunol. 1998; 28: 2197-2207.

Vignoud L, Albiges-Rizo C, Frachet P, and Block MR. NPXY motifs control the recruitment of the alpha-5/beta-1 integrin in focal adhesions independently of the association of talin with the beta-1 chain. J. Cell Sci. 1997; 110: 1421-1430.

Wei Y, Yang X, Liu Q, Wilkins JA, and Chapman HA. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. J. Cell. Biol. 1999; 144: 1285-1294.

Westmeyer A, Ruhnau K, Wegner A, and Jockusch BM. Antibody mapping of functional domains in vinculin. EMBO J. 1990; 9: 2071-2078.

Ylanne J, Chen Y, O'Toole TE, Loftus JC, Takada Y, and Ginsberg MH. Distinct functions of integrin alpha and beta subunit cytoplasmic domains in cell spreading and formation of focal adhesions. J. Cell Biol. 1993; 122: 223-233.

Zent R, Fenczik CA, Calderwood DA, Liu S, Dellos M, and Ginsberg MH. Class- and splice variant-specific association of CD98 with integrin beta cytoplasmic domains. J. Biol. Chem. 2000; 275: 5059-5064.

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