Cytoplasmic tails of integrin αIIbβ3 in the regulation of integrin activation, cell adhesion and spreading

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By

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ABSTRACT

Integrins are major adhesion receptors for the extracellular matrix (ECM). This thesis focuses on the motifs and interactions within integrin cytoplasmic tails during integrin-mediated cell adhesion and spreading. The present study investigated the significance of the skelemin-αIIbβ3 interaction using Chinese Hamster Ovary (CHO) cells expressing wild-type or mutant αIIbβ3 receptors defective in skelemin binding. Most mutant cells displayed unimpaired adhesive capacity and spreading on immobilized fibrinogen at the early stages of cell spreading. In addition, they formed normal focal adhesions and stress fibers with no indication of impaired cell spreading. K716A, and H722A mutant cells exhibited the greatest cell spreading, which was associated with enhanced p-Src activation. The K716 residue appeared to be the most important for skelemin binding in previous in vitro studies. Here, the protrusions of the leading edge of K716A cells showed strong colocalization of talin with αIIbβ3 which was associated with a loss in skelemin binding. These data suggest that the binding of skelemin to αIIbβ3 is not essential for normal cell spreading, but may act to exert contractile forces on cell spreading and coordinate the binding of talin to the membrane proximal region of integrin tails. The functional mode of peptides corresponding to the central motifs of the αIIb and αV tail, KRNPPPLEED (αIIb peptide) and KRVRPPQEEQ (αV peptide) was also investigated. Both peptides inhibited Mn2+-activated αIIbβ3 binding to soluble fibrinogen as well as the binding of αIIbβ3-expressing CHO cells to immobilized fibrinogen. Breast cancer progression has been linked to tumor cell interaction with ECM. Our αIIb and αV peptides also inhibited adhesion of two breast cancer cell lines (MDA-MB-435 and MCF7) to αV integrin ECM ligand vitronectin. Replacement of RPP with AAA significantly attenuated the inhibitory activity of the αIIb peptide. β-tubulin was identified as a potential αIIb peptide-binding partner, suggesting that microtubule cytoskeleton may participate in the regulation of integrin functions. These results provide insights into the mechanisms by which the central motifs of αIIb and αV tail regulate integrin activation and integrin-mediated cell adhesion.
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DEDICATION

To my dear parents

Hongyuan Li

Guixiu Che

献给我亲爱的父母

*For their constant support, encouragement, care, and unfailing love*

因为他们不断的支持，鼓励，关怀和无尽的爱
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<th>Full Form</th>
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<tr>
<td>ACSs</td>
<td>acute coronary syndromes</td>
</tr>
<tr>
<td>ADMIDAS</td>
<td>adjacent metal ion dependent adhesion sites</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CIB</td>
<td>calcium- and integrin-binding protein</td>
</tr>
<tr>
<td>CSK</td>
<td>c-Src kinase</td>
</tr>
<tr>
<td>CT</td>
<td>cytoplasmic tail</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>Dok1</td>
<td>docking protein 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EPI</td>
<td>epinephrine</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>$F_{Ca}^{+}/F_{EDTA}^{+}/F_{Mn}^{2+}$</td>
<td>the mean fluorescence intensity (MFI) of PAC-1 binding in the presence of Ca$^{2+}$, EDTA, or Mn$^{2+}$</td>
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FAK  focal adhesion kinase
FBS  fetal bovine serum
FL1  cell fluorescence intensity before washing in cell adhesion assay
FL2  cell fluorescence intensity after washing in cell adhesion assay
Fmoc  fluorenlymethyl-carbonyl
G418  geneticin
GAPs  GTPase activating proteins
GEFs  guanine nucleotide exchange factors
GFP  green fluorescence protein
GPCRs  G-protein-coupled receptors
HBTU  O-benzo triazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate
HOBt  N-hydroxybenzotriazole
HPLC  high-performance liquid chromatography
ILK  integrin linked kinase
IMC  inner membrane clasp
LAD I  leukocytes adhesion deficiency type I
MFI  mean fluorescence intensity
MIDAS  metal ion dependent adhesion site
MMP  matrix metalloproteinase
MRLC  myosin regulatory light chain
NMR  nuclear magnetic resonance
NOE nuclear overhauser effect
NHS-Biotin N-Hydroxysuccinidobiotin
OMC outer membrane clasp
PAC-1 a $\alpha_{IIb}\beta_3$ activation-specific antibody
PBS phosphate buffered saline
PRP platelet-rich plasma
PPP Platelet-poor plasma
PCI percutaneous coronary intervention
PE phycoerythryn
PKC protein kinase C
PMA phorbol myristate acetate
PMSF Phenylmethysulfonyl Fluoride
PP2A protein phosphatase 2A
$\alpha/\beta$ PS Drosophila position-specific (PS) genes encode $\alpha$ or $\beta$ integrin ortholog
PTB phosphotyrosine-binding
PTP protein–tyrosine phosphatase
SDS sodium dodecyl sulphate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
SkIgC4 skelemin immunoglobulin C2-like repeats 4
SkeC2 skelemin immunoglobulin C2 motifs 4-5 that contain the $\alpha_{IIb}\beta_3$-binding domain
<table>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>Src</td>
<td>a non-receptor tyrosine kinase encoded by the proto-oncogene of src which is highly similar to the v-src gene of Rous sarcoma virus</td>
</tr>
<tr>
<td>SyMBS</td>
<td>synergistic metal ion binding site</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline and tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Integrin functions

Integrins are type I transmembrane proteins consisting of noncovalently linked α and β subunits. In mammals, 18 α subunits pair with 8 β subunits to form a large integrin family with at least 24 distinct integrin heterodimers (1) (Figure 1.1).

Figure 1.1: The integrin family members. In vertebrates, the integrin family contains 24 heterodimers. They are divided into several subfamilies based on evolutionary relationships (coloring of α subunits), ligand specificity and leukocyte-specific expression in the case of β2 and β7 integrins. Adapted from reference (1).
Integrins are essential adhesion receptors that mediate cell-cell and cell-matrix interactions. Their primary function is to mediate cell adhesion, which provides a physical support for a cell and various tissue types. Each integrin family member can bind with multiple ECM proteins, such as bone matrix proteins, vitronectin, collagens, fibronectins, fibrinogen, laminins, thrombospondins, and von Willebrand factor. Integrins also bind with particular motifs or ligands. The preference of integrin binding is determined by the affinity, availability and conformation of ligands in the microenvironment (2). Cells expressing particular integrins can adhere or migrate toward a specific region where integrin ligands are available. Integrin attachment of cells to the ECM is important for the developmental processes (3). Genetic deletion studies with *Drosophila melanogaster* which have lower integrin redundancy suggested that disruption of αPS or βPS [Drosophila position-specific (PS) genes encode α or β integrin ortholog] resulted in embryonic lethal or defective tissue morphogenesis, such as muscle detachment, dorsal closure defects, delayed gut migration, and blisters in the wing (3).

Integrin-mediated cell-cell adhesion is facilitated by the β2 family. β2 integrins are leukocyte-specific receptors and bind cellular receptor ligands such as vascular cell adhesion molecule-1 (VCAM-1) and/or intercellular cell adhesion molecule (ICAM) on endothelial cells (1, 4). β2 integrins are important for many adhesion-dependent processes, including chemotaxis, phagocytosis and homotypic aggregation. The lack of expression of β2 integrins or the expression of dysfunctional β2 integrins lead to leukocyte adhesion deficiency type I (LAD I) disease, a life-threatening condition.
associated with recurrent microbial infections with associated neutrophilia (5).

Not only can integrins mediate physical attachment of cells to their environment, but they can also be a primary component of sensory machinery for cells (6). By binding with their ligands, integrins recognize and sense environmental cues, including chemical or physical stimuli, and transmit these signals into cells. Diverse signalling pathways can be initiated and transmitted by integrin, which influence varieties of cell behaviours, such as cell adhesion, migration, proliferation, differentiation, and apoptosis. These basic biological events determine processes of embryonic development, tissue repair, homeostasis, and immune responses. Abnormal integrin function is linked to the progression of some diseases, such as thrombosis, autoimmune disease, and tumour metastasis.

Our lab is particularly interested in integrin α_{IIb}β_{3}, expressed on platelets, megakaryocytes, basophils, mast cells and some tumour cells (7). α_{IIb}β_{3} is the most abundant integrin expressed on platelets and responsible for platelet aggregation and normal homeostasis. When α_{IIb}β_{3} is activated, it binds to multivalent fibrinogen and von Willebrand factors in plasma, which then bridge to other α_{IIb}β_{3} molecules on adjacent platelets leading to platelet-platelet interaction. Genetic defects in integrin α_{IIb} or β_{3} subunit results in Glanzmann thrombasthenia, a bleeding disorder (8).

1.2 Integrin activation

Integrins are normally expressed on cells in an inactive state, and are unable to productively bind to their ligands. This is particularly important for immune cells and
platelets circulating in the bloodstream, as integrins must be in a quiescent state under physiological conditions. Stimuli such as cytokines and coagulation factors can quickly cause extracellular changes of integrins, switching integrins from a low ligand binding affinity state to a high affinity state (a process known as inside-out signalling). αIIbβ3 on platelets represents a typical model to investigate integrin activation. αIIbβ3 in the low-affinity state does not interact with soluble fibrinogen under physiological conditions. After vascular injury, stimulation by a number of agonists (such as thrombin, ADP, epinephrine) on platelets changes the αIIbβ3 conformation and increases its affinity to fibrinogen. How these agonists initiate integrin activation (inside-out signalling pathway) is not completely clear, but a recent study suggested that platelet agonists can bind to specific G-protein-coupled receptors (GPCRs) in platelets, and the heterotrimeric G-protein may play a key role in translating the signal from GPCRs to integrin αIIbβ3 (9). Binding of fibrinogen to αIIbβ3 on platelets results in platelet aggregation. This quick and precisely controlled process is critical in order to prevent blood loss at the site of a wound, while maintaining normal blood flow. Abnormal αIIbβ3 activation leads to potentially life-threatening thrombosis and is involved in cardiovascular diseases.

Another example of the control of integrin activation is integrin αvβ3, which is widely expressed in diverse cell types. αvβ3 is used as a marker of angiogenic vascular tissue as quiescent endothelial cells expressed low levels of αvβ3 but endothelial cells in angiogenic vessels have increased αvβ3 expression (10). Activation of αvβ3 can be induced by agonists, such as ADP, phorbol myristate
acetate (PMA) and Mn$^{2+}$. PMA is also a potent activator of protein kinase C (PKC) (11). Previous work found that the PKC pathway is important for the inside-out signalling events that activate $\alpha_\nu \beta_3$ (11, 12). In addition, the neutral protease calpain, a secondary signalling molecule in cells, is also required for the modulation of $\alpha_\nu \beta_3$ activation (11). Calpain cleaves a variety of substrates of multiple intracellular signalling pathways, including PKC (13). Moreover, $\alpha_\nu \beta_3$ has been implicated in tumour invasion and metastasis of several tumour types, such as glioma, breast cancer, and melanoma (14, 15). In breast cancer, the expression of $\alpha_\nu \beta_3$ on tumour cells, particularly in its high affinity state, promotes tumour progression and metastasis. Over-expression of the constitutively active mutant $\alpha_\nu \beta_{3D723R}$, but not wild-type $\alpha_\nu \beta_3$, in MDA-MB 435 cells resulted in a significant increase in metastatic activity in mouse models (16, 17). The drastic increase in metastatic activity of active $\alpha_\nu \beta_3$ has been attributed to its capability of supporting tumour cell arrest during blood flow through interaction with platelets, up-regulation of the mature form of metalloproteinase-9 (MMP-9) which maximizes tumour cell mobility, and efficient metastasis growth in metastatic tissue through continuous up-regulation of vascular endothelial growth factor (VEGF) (16, 18, 19).

1.3 Integrin structure and the conformational change during integrin activation

As a typical integrin, $\alpha_{IIb}\beta_3$ is a heterodimer composed of $\alpha_{IIb}$ and $\beta_3$, which are held together by numerous non-covalent protein-protein and protein-metal ion contacts. Each subunit has a large extracellular domain, a single-pass transmembrane
domain, and a short cytoplasmic tail.

1.3.1 Extracellular domain

The extracellular domain of integrin is a ligand binding region. The first X-ray crystal structure of the full-length extracellular domain of integrin was obtained from \( \alpha_v\beta_3 \) and its complex with the RGD peptide ligand (20, 21). The structure of \( \alpha_v\beta_3 \) revealed the inter-subunit interface, ligand binding sites, some previously unpredicted modules, and novel calcium-binding sites. Ligand binding sites form a globular headpiece which is followed in each subunit by two long legs. The two subunit interface also lies within the head between the seven-bladed \( \beta \)-propeller in \( \alpha \) subunits and the \( \beta \)A-domain in \( \beta_3 \) subunits (Figure 1.2). The \( \beta \)-propeller domain of the \( \alpha \) subunit contains Ca\(^{2+} \)-binding motifs that are important for formation of the inter-subunit interface. In the absence of a ligand, the structure folds into an extremely bent conformation and ligand binding induces an extended conformation as shown in Figure 1.2. Electron microscopy studies of \( \alpha_v\beta_3 \) reconstituted in lipid bilayers showed an extended conformation, especially when integrin was activated by Mn\(^{2+} \) and small-molecule ligands (22, 23). Therefore, there is a general acceptance that a bent structure represents an inactive state and that the extended structure exposes ligand binding sites and defines the active state.

The crystal structures of \( \alpha_{IIb}\beta_3 \) with or without a bound ligand have also been resolved (24, 25). The \( \alpha_{IIb}\beta_3 \) structure is very similar to that of \( \alpha_v\beta_3 \) as shown in Figure
Figure 1.2: Ribbon schematic of crystallized αvβ3 in a bent (left) and extended conformation (right). αv is shown in blue and β3 in red. Its 12 known and novel domains assemble into a globular “head” and two “tails”. Adapted from reference (21).

1.2. However, an intermediate form of αIIbβ3 between the bent and extended form was reported in a cryo-electron microscopy (cryo-electron microscopy: imaging specimens in a transmission electron microscope under cryogenic conditions) study (26). The bent conformation of αIIbβ3 incorporated into liposomes was also observed in the presence of Mn2+ (27). Quantitative analysis of electron microscopic images of
lipid-embedded $\alpha_{\text{IIb}}\beta_3$ revealed that activation of integrin shifted the conformational equilibrium in favour of the extended form that leads to an increase in ligand binding affinity (28).

Three metal binding sites in the $\beta$A domain of the $\beta_3$ subunit support the ligand binding site (24). They are occupied by the divalent cations Ca$^{2+}$ and Mg$^{2+}$. A metal ion dependent adhesion site (MIDAS) in the $\beta$A-domain binds with Mg$^{2+}$ and participates in ligand binding. MIDAS is flanked by two Ca$^{2+}$ binding sites, named the adjacent metal ion dependent adhesion site (ADMIDAS) and the synergistic metal ion binding site (SyMBS) (24). ADMIDAS binds an inhibitory Ca$^{2+}$ ion and replacement of Mn$^{2+}$ at this site results in a structural change that produces an active integrin (29).

1.3.2 Transmembrane domain

The transmembrane domains (TMDs) of integrins are single membrane-spanning structures, comprised of about 20 amino acid residues, and are highly conserved across integrins. The TMD of integrin $\alpha_{\text{IIb}}\beta_3$ is rich in hydrophobic sequences and closely resembles that of many of the other 18 $\alpha$ and 8 $\beta$ human subunits. Its C-termini are located close to lysine residues K989 in $\alpha_{\text{IIb}}$ and K716 in $\beta_3$, which begin the cytoplasmic regions. The TMD domain is critical for integrin activation and for transmitting signals across the plasma membrane. Studies of nuclear magnetic resonance (NMR) structure (26, 30, 31), disulfide bond scanning (32), and Leu substitution (33) found that there is an $\alpha$-$\beta$ heterodimeric TMD interaction in integrin
in its resting state. Dissociation of the α-β interaction at the TMD triggers receptor activation and signalling (33-35).

NMR is a useful tool to study a weak and dynamic complex. By using NMR, most of the integrin TMD and cytoplasmic tail structural data were obtained in a variety of membrane mimetic solvents. NMR structures of the αIIbβ3 TMD show that the αIIb TMD adopts a short and straight helix structure, whereas the β3 TMD forms a long and tilted helix, which extends to the intracellular side and forms a continuous helix with the membrane-proximal region of β3 cytoplasmic tails (32, 36). At the N-terminus of the β3 TMD, Gly residues at 708, 976 and 972 allow close interhelical packing, which explains the observation that mutational substitution of either Gly residue with a bulky amino acid results in constitutively active integrin (33). This helical TMD packing is referred to as the outer membrane clasp (OMC) that stabilizes the low-affinity state of the integrin (31).

1.3.3 Cytoplasmic tails: structures and interactions

Integrin cytoplasmic domains are generally short, but vital in regulating integrin ligand-binding competency and signalling functions. The cytoplasmic tails are highly similar between αIIb and other integrin α-subunits, which are composed of a highly conserved N-terminus followed by a variable C-terminus (Figure 1.3).

Early mutational studies show that deletion of almost the entire αIIb-cytoplasmic tail (G991–E1008) or deletion of α or β membrane-proximal regions constitutively activates αIIbβ3 (37, 38). Therefore, the membrane-proximal regions of αIIbβ3 tails were
Figure 1.3: Sequence alignment of various human integrin α-cytoplasmic domains. The sequence identity and similarity are highlighted in red and purple. Adapted from reference (4).

suggested to negatively regulate integrin activation (38). The opposing charged Arg995 in αIIb and Asp723 in β3 were proposed to form a salt bridge (39). Charge reversal of either one of these residues (R995D or D723R) or substitution of either one with non-charged residues (for example, R995A) resulted in the expression of a constitutively active αIIbβ3. However, when both charges were reversed, the integrin stayed inactive (39). These studies indicate that the electronic interaction between membrane-proximal regions of αIIb or β3 maintains integrins in an inactive state, while breaking this interaction makes the integrin active (39). The interaction between αIIb and β3 cytoplasmic peptide can be detected by surface plasmon resonance (SPR).
Their heterodimerization occurred in a 1:1 stoichiometry with a weak affinity in the micromolar range, which was impaired by the R995A substitution or deletion of KVGFFKR in αIIb (40).

The structures of αIIbβ3 cytoplasmic tails have been characterized under heterodimeric conditions (41-44). Most structural studies of αIIbβ3 identified electrostatic and hydrophobic interactions in the membrane proximal regions, which support the previous mutational studies. The interaction occurred between the conserved 990GFFKR995 in αIIb subunits and the conserved 722HDRKE726 in β3 tails. The current view is that both the interactions through the membrane-proximal regions and packing of TMDs are required to keep the resting integrin receptor in an inactive state and disrupting either interaction activates integrins (35, 45). Evidence for this view comes from the mutational study showing that the individual effects on integrin activation produced by mutations in the TMD or tail interface were weak, whereas the combination of the two mutations had a strong synergistic effect (36).

The membrane distal region of the αIIb cytoplasmic tail has a significant structural feature, described as a turn formed at RPP999. The NMR structure of a synthetic peptide encompassing the αIIb cytoplasmic tail reveals a “closed” conformation (Figure 1.4A) (4). The highly conserved N-terminal membrane-proximal region forms an α-helix followed by a turn at PP which allows the acidic C-terminal loop to fold back and interact with the positively charged N-terminal helix. Replacing the double prolines with alanines (P998A/P999A) resulted in misfolding of αIIb (Figure 1.4B). Although the N-terminal α-helix is retained in the mutant peptide, the double mutation
disrupts the turn, thereby preventing interactions between the N-terminal helix and C-terminal region and resulting in an “open” conformation. The structure of the mutant is apparently less rigid, with a significantly reduced number of Nuclear Overhauser Effect (NOE) constraints in its NMR signals as a result of disruption of N- and C-terminal contacts. Furthermore, mutation of $\alpha_{IIb}\beta_3$ (P998A/P999A) expressed in CHO cells rendered $\alpha_{IIb}\beta_3$ constitutively active to bind extracellular ligands, resulting in fibrinogen-dependent cell-cell aggregation (46).

Figure 1.4: Structural highlights of the cytoplasmic domain of $\alpha_{IIb}$. (A) Backbone of wild type $\alpha_{IIb}$ showing the residues participating in the loop structure formation between the N-terminal helix and C-terminal region. The interactions between the N and C termini are primarily through an electrostatic network between the side chains of K994, R997, E1001, D1003, D1004, and E1005. (B) Backbone overlay of wild-type $\alpha_{IIb}$ (purple) and mutant $\alpha_{IIb}$ (yellow) showing the structural difference. Adapted from reference (4).
One possible reason for the activation of the mutated α₁bβ₃ (P998A/P999A) is because the downstream activators can bind to the integrin tails when they are “open” (Figure 1.4) (4). Multiple proteins have been identified that bind to α₁b tails. For example, the α₁b cytoplasmic tail interacts with the chloride channel ICln, the calcium- and integrin-binding protein (CIB), and the catalytic subunit of protein phosphatase 1 (47-50). The binding of these cytoplasmic proteins may serve to regulate integrin activation and function. However, almost all of the proteins were reported to bind to the membrane proximal region of α₁b tails. Therefore, searching for proteins that interact with the central turn motif of α₁b tails may provide useful insights for understanding the significance of the turn structural feature which still remains uncertain.

Compared with α₁b tails, the structure and function of β₃ integrin tails have been more extensively studied. There are two well-defined motifs within β integrin tails: the NPₓY⁷⁴⁷ motif (x stands for any amino acid and is a leucine in the β₃ tail) and the NₓₓY⁷⁵⁹ motif (xx is isoleucine and threonine in the β₃ tail). These motifs represent recognition sequences for phosphotyrosine-binding (PTB) domains (51). NPₓY⁷⁴⁷ serves as the binding site for talin, a well-established integrin activator. Talin binding has previously been assumed to be the final and common step of integrin activation (52, 53). The PTB domain within the head domain of talin binds to integrin β₃ tails with much higher affinity (Kd ~ 100nM). Y⁷⁴⁷ of NPLY⁷⁴⁷ within β₃ tails is necessary for this interaction (54, 55). An NMR study showed that the talin-binding site on the β₃ tail extended to the membrane proximal region, completely overlapping the region
of β3 interaction with αIib. Adding a talin head destroyed the NMR signals of a αIib-β3 tail interface (43). Reconstitution of physiological integrin activation with nanodiscs and a single lipid-embedded integrin showed that talin binding alone was sufficient to activate integrin αIibβ3 (28). These studies are consistent with the model that the membrane-proximal regions form the inner membrane clasp (IMC) that maintains the low-affinity state, and breaking this clasp induces integrin activation (31).

However, it is interesting that although many PTB-domain-containing proteins bind integrins, only talin exhibits the capacity to activate integrins. A further study found that a second interaction happens between talin and the membrane proximal helix of β3 tails, which causes α and β tail separation and in turn the extended and active conformation of the integrin extracellular domain (56). Several integrin activation inhibitors with PTB domains have been identified. These inhibitors, such as docking protein 1 (Dok1) and filamin, can bind to the β3 NPLY747 motif through its PTB domain but do not activate integrin, and thereby can function as competitive inhibitors of talin binding (51, 57).

Recently, another protein, kindlin, has been identified as a direct integrin activator. Platelets lacking kindlin-3, which is specific to hematopoietic cells, were unable to activate integrins despite normal talin expression (58). Kindlin-1 and kindlin-2 can function as co-activators of talin and enhance the ability of overexpressed talin head domain to activate integrin αIibβ3 in endothelial cells and CHO cells (59, 60). Their binding to β3 tails requires the β3 C-terminal region (NITY759) (59, 60).

1.3.4 Integrin clustering
In addition to conformational changes of individual integrins, integrin activation promotes the lateral assembly of integrins (integrin clustering) within the plane of the plasma membrane. Integrin conformational changes and clustering are complementary for a cell to bind strongly to the ECM and for integrin signalling (61). Ligand affinity of individual integrins is increased by integrin activation, but for a cell to bind strongly to the ECM, it is not enough. Integrin clustering increases the avidity of binding so that the sum of hundreds or thousands of weak interactions constitute a tightly bound adhesive unit (3). In addition, integrin clustering is important for integrin outside-in signalling, increasing the local concentration of integrin-associated signalling molecules and triggering the activation of Src (a non-receptor tyrosine kinase encoded by the proto-oncogene of src which is highly similar to the v-src gene of Rous sarcoma virus) and FAK (focal adhesion kinase), as discussed in section 1.5.3.2 of this chapter.

\( \alpha_{Ib}\beta_3 \) clustering is promoted by extracellular and intracellular factors, including the binding of multivalent ligands, ligand self-association, relief from cytoskeletal constraints, and homomeric interactions of the transmembrane domains (62-65). Optimum oligomerization requires the participation of the integrin's transmembrane and cytoplasmic regions (66). However, whether the activated integrins can cluster spontaneously remains controversial. Protein fragments encompassing the transmembrane helix plus cytoplasmic tails of the \( \alpha \) and \( \beta \) subunits of \( \alpha_{Ib}\beta_3 \) were able to form homodimers or homooligomers in phospholipid micelles (67, 68). It is possible that integrin TMDs interact heteromerically in the inactive state, but when
integrins are activated, the transmembrane domains separate and homooligomerize, which drives lateral clustering (62). However, cysteine scanning mutagenesis of integrin αIIbβ3 transmembrane domains did not reveal a specific interaction of these domains after integrin activation in living cells (69). Electron microscopic images of purified and activated αIIbβ3 integrins that were reconstituted into lipid bilayers did not show spontaneous clustering, but integrins clustered after fibrinogen binding (22). In a study using β-gal complementation and bioluminescence resonance energy transfer (BRET) assays to detect integrin clustering, direct activation of αIIbβ3 by MnCl2 or an activating antibody did not cause clustering in the absence of fibrinogen (64).

1.4 Integrin outside-in signalling

Once integrins are active and clustering, they can transmit information into cells, a process known as outside-in signalling. These intracellular changes can be divided into three temporal stages: immediate, short-term and long-term (3). Immediate intracellular changes include rapid phosphorylation events, particularly tyrosine phosphorylation of specific substrates, and an up-regulation of lipid second messengers such as phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P2) and phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3). Short-term changes consist of cytoskeletal rearrangements, which are required for cell spreading, migration and polarity establishment. This step usually occurs within one hour of cell adhesion, and involves dynamic connections of integrins to the cell cytoskeleton, and activation of kinases and signal transduction pathways to regulate cytoskeletal rearrangements.
Long-term attachment to the ECM ultimately results in changes in signalling pathways and gene expression that influence the survival, growth, and differentiation of cells. Figure 1.5 shows the major signalling pathways and key players located downstream of integrin activation.

Figure 1.5: Integrin outside-in signalling. Major signalling pathways and key players downstream of integrin activation are illustrated. Integrin regulates downstream signalling pathways in cooperation with growth factor receptors to influence cell proliferation, differentiation, cell shape and migration. Integrin-associated proteins linking integrins and these signal transduction pathways are shown within the pink-purple pentagon beneath the clustered integrins. Integrins activate ERK and JNK to regulate cell proliferation. Integrins regulate Rho GTPase activity through the Src/FAK complex, resulting in cytoskeletal organization and regulation of cell motility. Integrins also activate PI3K and regulate cell survival through Akt. Adapted from reference (1).

The inside-out and outside-in signalling pathways constitute the bidirectional signalling across integrin receptors. Cytoplasmic tails of integrins serve as receivers
and transmitters of the bidirectional signal. Integrin cytoplasmic tails possess no enzymatic or kinase activity. One of the primary mechanisms of signal transduction is via recruiting cytoplasmic binding proteins following integrin activation and clustering. An overwhelming collection of cytoplasmic molecules that directly or indirectly interact with $\alpha_{Ib}\beta_3$ has been identified (70, 71).

1.5 Integrin mediated cell adhesion and spreading

1.5.1 Integrin adhesome

Integrins mediate cell-matrix adhesion by linking the ECM to the actin cytoskeleton (1). First, ligand-bound integrins cluster together and engage other proteins to organize into small and transient cell-ECM contacts called nascent adhesions (72, 73). As cells spread and migrate, nascent adhesions either disassemble or progress to larger dot-like adhesions called focal complexes, which locate slightly back from the leading edge and persist for several minutes (74). In more fully spreading cells, focal complexes can mature into larger and elongated focal adhesions. Focal adhesions reside at the ends of actin stress fibers (bundles of actin filaments) (74). Focal adhesions and stress fibers are cellular structures that are important for exerting contractile forces during cell migration.

Adhesion sites (nascent adhesions, focal complexes and focal adhesions) are large multiprotein complexes consisting of at least 180 signalling, structural, and adaptor molecules (75). These molecules and their interactions (at least 742) form a complex network termed the ‘integrin adhesome’ (75). In general, the integrin adhesome is
divided into structural and regulatory/signalling sub-networks (75, 76). The structural sub-network serves as a mechanical linkage between the ECM and cell cytoskeleton and generates traction force required for cell spreading and migration (7). Integrins anchor the intracellular cytoskeleton to the ECM by recruiting cytoskeletal proteins, such as talin, filament, α-actinin and tensin (76). These proteins can bind to F-actin and therefore couple integrins to the cytoskeleton directly (70). The regulatory/signalling sub-network includes multiple biochemical signalling hubs for many cellular processes, including actin polymerization and actomyosin contraction, cell proliferation and survival, and gene expression (77, 78). Integrins recruit and activate various catalytic proteins, such as FAK, integrin linked kinase (ILK), Src family kinase (SFK) and protein phosphatase 2A (PP2A). They facilitate the propagation of signal-transduction pathways from adhesion sites, and translate environmental cues into biochemical signals within cells (70).

1.5.2 Cell spreading: integrin-mediated control of cell protrusion and contraction

Cell spreading on the ECM requires two fundamental cellular processes, actin polymerization and actin-myosin contraction, the balance of which are tightly controlled and organized by integrin outside-in signalling (79, 80). After spherical cells in suspension adhere to the ECM, they quickly spread to a more flattened shape. They form protrusions at the cell leading edge, broad lamellipodia and spike-like filopodia. Formation of cell membrane protrusions is driven by actin polymerization (81). Actin polymerization is catalyzed by the ARP2/3 complex, which makes a
branched actin filament network through its actin nucleation function (82). After spreading, the cells form specialized structures such as stress fibers and focal adhesions. Contraction of actin stress fibers is mediated by myosin. During contraction, actin filaments do not shorten, but myosin mediates actin filaments to slide past each other (83).

The balance between actin-mediated protrusions and myosin-mediated contractions is controlled by members of the Rho GTPase family, particularly Rac, RhoA, and CDC42 (77, 84). The activity of ARP2/3 is regulated by Rac and CDC42 (72, 85). Myosin activity is regulated by phosphorylation of its myosin regulatory light chain (MRLC) at Thr18/Ser19 (86). Phosphorylation of MRLC is dependent on several protein kinases and phosphatases, most of which are downstream effectors of Rho GTPases (72).

As initial cell adhesion and spreading occurs, Rac and CDC42 are activated at the cell leading edge to mediate the formation of actin-rich membrane protrusions (80). Rac and CDC42 probably have overlapping functions to enhance protrusion formation. Whereas the expression of activated CDC42 alone produces filopodia, and expression of Rac stimulates formation of broad lamellipodia (3), RhoA promotes cell contractility by increasing phosphorylation of MRLC (87). At later stages of cell spreading, RhoA activation is necessary for maturation of focal adhesions and retraction of the cell rear edge (13, 80).

Two groups of molecules acting downstream of integrin outside-in signalling, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs),
directly control Rho GTPase activity (80). Rho family GTPases are active when they are GTP-bound and inactive when bound to GDP. Activation is catalyzed by GEFs and inactivation is promoted by GAPs that stimulate the intrinsic GTPase activity of the Rho proteins. In one example of this pathway, integrin ligation and clustering lead to activation of tyrosine kinases FAK and Src, which phosphorylate and recruit the scaffolding protein p130Cas (88). Tyr phosphorylation of p130Cas recruits SH2-containing adaptor protein Crk, which in turn recruits or activates Dock180. Dock180 is a GEF for Rac and mediates GTP loading and membrane targeting of Rac (89, 90). Active Rac promotes protrusion at the leading edge of the cell via WASP/WAVE-dependent activation of the Arp2/3 complex (91).

1.5.3 Integrin cytoplasmic tail-binding proteins

Integrin cytoplasmic tails play a critical role in regulating cell adhesion and spreading. Integrin cytoplasmic tails serve as a scaffold for adhesion components which in turn recruit other additional components leading to adhesome assembly (92, 93). Many binding proteins have been identified, but there are relatively few binding sites within αIIb and β3 cytoplasmic tails (47, 70, 71). For example, Src-family kinase Fyn (94), paxillin (95) and skelemin (96) can each bind to the membrane-proximal region of β3 cytoplasmic tails. The membrane-proximal region of β3 tails is also involved in the formation of the interface with αIIb tails in inactive integrins. Once integrins bind to their ECM ligands, unclasping the interface may unmask binding sites for these proteins and enable their recruitment (70).
1.5.3.1 Proteins linking integrins to the cytoskeleton

Talin is the best-studied protein that is recruited by integrin cytoplasmic tails into adhesions. Talin contains a globular head and a long rod domain. The head domain is sufficient for integrin activation by binding to integrin cytoplasmic tails (28). The talin rod contains many functional domains, including domains responsible for dimer formation, a direct actin-binding site at the carboxy-terminus, a second integrin-binding site and several binding sites for vinculin (97). Talin accumulates at nascent adhesions which form within 15 minutes after cells are plated on the ECM (98). Microinjection of antibodies to talin or talin antisense RNA disrupts stress fibers and inhibits cell adhesion, spreading and migration (99). The ECM–integrin–cytoskeletal linkage provides a migration force at the leading edge of a migratory cell. A previous study showed that the binding of talin to minimal complexes of ligated integrins is an early step in the formation of integrin-mediated ECM–cytoskeleton connections (100). After αvβ3 binding to fibronectin, a weak bond connecting fibronectin and cytoskeleton was established to provide the initial mechanical force (100). However, this bond was disrupted in fibroblasts deficient in talin expression (100). Reexpression of wild-type talin1, but not talin1 lacking the actin-binding site, restored the weak force (100).

Other cytoskeletal proteins including filamin, α-actinin, myosin, and skelemin are reported to directly bind with β3 tails (70, 101). Filamin and α-actinin are also actin-binding proteins. Filamin has important roles for actin crosslinking and is
involved in cell spreading and motility by connecting integrins to the cytoskeleton (57, 102). α-actinin is also an effective actin cross-linker (103, 104), and has essential roles in the assembly and maturation of nascent adhesions (105).

Skelemin is another cytoskeletal protein that can interact with integrin cytoplasmic tails (33). Skelemin is a myosin-binding protein and was originally found to concentrate at the periphery of M-disc (the region in the middle of myosin bundles) in muscle cells (106, 107). In vitro studies mapped the skelemin binding site at the membrane proximal regions of αIIb and β3 tails (96). It is of interest that skelemin co-localizes with αIIbβ3 in newly formed adhesions, but does not appear to be present in focal adhesions (108). However, the significance of skelemin binding is not yet clear.

1.5.3.2 FAK and Src: switching on integrin signalling

FAK and Src are crucial mediators of integrin signalling pathways that regulate the activation of Rho-GTPases. Both of them are non-receptor tyrosine kinases and their recruitment to the integrin cytoplasmic tail is an early event in adhesion assembly and acts to initiate integrin outside-in signalling.

The N-terminal domain of FAK binds to integrin-tail peptides in vitro (95). Ligand engagement and clustering of integrins results in rapid autophosphorylation of FAK at Tyr397, as well as several additional sites within the kinase and C-terminal domains (109). Phosphorylation at Tyr397 leads only to a limited activation of FAK, but it generates an SH2-mediated interaction with Src. This interaction enzymatically
activates Src by releasing autoinhibition imposed by the interaction between its SH2 domain and the inhibitory phosphorylated tyrosine (Tyr529). Src in turn phosphorylates other tyrosine sites of FAK, leading to maximal activation of FAK (109, 110).

FAK activation is closely associated with focal adhesion formation and cell migration (111). FAK-null fibroblasts exhibit enhanced paxillin phosphorylation and adhesion formation, suggesting FAK activity is essential for efficient focal adhesion turnover in cell migration (112). FAK inhibition could result in compromised cell mobility whereas enhanced FAK activity promotes cell migration (111).

The Src family of kinases contains nine members; Src, Fyn, Yes, Lck, Hck, Blk, Fgr, Lyn and Yrk, where Src is considered to be a principle player in αIIbβ3-mediated signalling (3). Src constitutively associates with αIIbβ3 in platelets (113-115). The association is mediated by interaction of the SH3 domain of Src with the four terminal residues of the β3 tail, 759YRG (115). This association is important for integrin functions. For example, disrupting the interaction of integrin αIIbβ3 with Src kinase, either by substitution of β3 Thr762 with Ala or by treatment with an RGT synthetic peptide into human platelets leads to decreased platelet spreading on fibrinogen (116, 117). A “β3 (Δ760-762)” knock-in mouse strain was generated, which lacked RGT residues of β3 tails necessary for the interaction with Src, but retained residues necessary for talin-dependent fibrinogen binding. The mice had variably prolonged tail bleeding times. Their platelets showed reduced spreading after plating on fibrinogen (118).
Activity of Src is negatively regulated by a phosphorylated tyrosine residue (Tyr529), which binds to the SH2 domain of Src leading to a closed conformation and inactivation of Src. Csk (c-Src terminal kinase) is a negative regulator of Src kinases, acting by phosphorylating Tyr529 to maintain Src autoinhibition. Deposphorylation by protein-tyrosine phosphatase (PTP) at Tyr529 and phosphorylation at Tyr418 leads to Src activation. In resting platelets, Src forms a complex with αIIbβ3 and Csk (113). Fibrinogen binding to αIIbβ3 leads to Csk dissociation from αIIbβ3, PTP-1B recruitment to this inhibitory complex to dephosphorylate Tyr529, and autophosphorylation at Tyr418 (113-115). The clustering of integrins brings Src molecules into close proximity, potentially promoting Src transactivation. Another possible mechanism of Src activation is via FAK. Upon autophosphorylation, FAK Tyr 397 constitutes a docking site for the Src SH2 domain and relieves the intramolecular, inhibitory interactions on Src to activate the protein (119).

Src is required for integrin-mediated adhesion and spreading. Pharmacological and genetic inhibition of Src in a variety of cell types impaired adhesion, while exogenous expression of activated Src promoted cell adhesion and spreading (91). For example, in Src-deficient fibroblasts, cell spreading is inhibited on fibronectin or vitronectin. The defective spreading is rescued by re-expression of Src (120, 121). In a study using epithelial cancer cell lines as a model, elevation of Src expression and activation has been implicated in progression of cancer cells to metastatic cells, which is not linked to enhanced cell growth, but rather to enhanced cell attachment to ECM and assembly of adhesion complexes (122).
Src mediates Tyr phosphorylation of FAK, p130CAS, paxillin and other adhesion molecules (123, 124). Characterization of these proteins revealed that they are major components of integrin-dependent signalling pathways. As previously described, Src phosphorylates FAK leading to full activation of FAK, and Src and FAK function as a complex to initiate integrin downstream signalling (91). p130CAS and paxillin are scaffold proteins, in which the phosphorylation of their Tyr residues recruits and organizes signalling molecules.

1.5.4 Physiological and pathological implications

Platelets and αIIbβ3-expressing CHO cells adherent to fibrinogen are broadly used model systems to study cell adhesion and spreading. Upon activation by inside-out signalling, αIIbβ3 integrins on platelets are capable of binding to soluble fibrinogen to form a thrombotic clot. During this process, αIIbβ3 integrins not only mediate platelet-platelet interactions, but also transmit an array of signals into the platelets, inducing a series of coordinated events including platelet adhesion, shape change, spreading on fibrinogen or fibrin matrix and clot retraction (125). First, αIIbβ3 adhesion to fibrin stimulates dramatic cell shape changes. Discoid platelets develop spike-like filopodia and lamellipodial extension to mediate full spreading over the damaged area and thus seal the vessel wall (88). The morphologic changes are associated with dynamic actin polymerization and reorganization. After platelets adhere and aggregate at the wound site, the next step is the retraction of clots through contractile forces generated by the platelet actomyosin network and to ‘shrink’ the
thrombus' size (125). Retraction of clots stabilizes platelet aggregates, restores blood flow and also draws the wound edges together during wound repair. \( \alpha_{IIb}\beta_3 \) outside-in signalling leading to cytoskeletal reorganization and development of contractile force is crucial for these processes (126).

Tumor progression has been associated with altered integrin-mediated adhesion. To undergo metastasis, circulating tumour cells must attach to vascular endothelial cells or components of the vessel wall. They enter the blood flow to reach the target organs, and then colonize and grow in distant organs. Specific interactions of tumour cells with the favourable environments of distant organs help initiate and promote tumour metastasis and growth (127). Highly tumorigenic breast cancer cells expressing \( \beta_1 \) and \( \beta_3 \) integrin family members adhere rapidly to bone ECM, suggesting an important contribution of integrin-matrix interaction to the establishment of breast cancer cells in bone (128). Furthermore, integrin adhesion to the ECM provides the traction force necessary for tumour cell motility and invasion of distant organs (129). Intravenous inoculation of MDA-MB-231 transfectants overexpressing \( \alpha_v\beta_3 \) in animals increased bone metastasis when compared with the inoculation of mock-transfected cancer cells (130). Treatment of animals with antagonists of \( \alpha_v\beta_3 \) reduces bone metastasis (130, 131). Integrins also regulate ECM remodelling and protease activity such as matrix metalloproteinase (MMP), and support tumor cell proliferation in either adhesion-dependent or independent mechanisms (129).
1.6 $\alpha_{\text{IIb}}\beta_3$ antagonism

Excessive platelet aggregation is a key event in myocardial infarction and other thrombotic diseases. $\alpha_{\text{IIb}}\beta_3$ has become an attractive pharmacological target for antithrombotic therapy because of its importance in platelet aggregation. A number of $\alpha_{\text{IIb}}\beta_3$ antagonists have undergone clinical testing for their potential use as short-term and long-term cardiovascular therapeutics. In the 1990s, three intravenous $\alpha_{\text{IIb}}\beta_3$ antagonists approved by the FDA were: abciximab, a mouse/human chimeric antibody fragment c7E3 Fab; eptifibatide, a snake venom disintegrin-derived cyclic peptide; and tirofiban, a RGD peptidomemetic. These inhibitors have been approved for and are widely used for treatment of patients with acute coronary syndromes (ACSs), and have especially benefited patients undergoing percutaneous coronary intervention (PCI). The major side effect of these agents is bleeding complications. Choosing an optimal dosing was a challenge, as the window between the therapeutically efficacious doses to prevent platelet aggregation and higher doses that can lead to bleeding is narrow (132).

To achieve long-term suppression of platelet aggregation and to prevent cardiovascular diseases, orally active $\alpha_{\text{IIb}}\beta_3$ antagonists were developed. However, the clinical trials were disappointing, with lack of efficacy and increased mortality of patients with ACS (133). One potential contributing factor is the activating property of the $\alpha_{\text{IIb}}\beta_3$ antagonists. An early study in 1991 showed that RGD peptide binding could induce a high affinity fibrinogen-binding conformation of $\alpha_{\text{IIb}}\beta_3$ (integrin activation) and subsequent platelet aggregation (134). Several $\alpha_{\text{IIb}}\beta_3$ antagonists are
ligand-mimetics, suggesting they may possess partial agonist effects. The monoclonal antibody abciximab, when used at low concentrations, also demonstrated an intrinsic activating property, resulting in fibrinogen binding to $\alpha_{IIb}\beta_3$ and activation of platelets \((135)\). Specific types of $\alpha_{IIb}\beta_3$ antagonists can augment agonist-induced release of $\alpha$-granules, a marker of platelet activation \((136)\). In fact, platelet activation has been reported in patients with ACS receiving oral $\alpha_{IIb}\beta_3$ antagonist at low concentrations \((137)\).

An alternative strategy is to target the intracellular events of integrin signalling. Mice harbouring the point mutation (L746A) in the $\beta_3$ tail that selectively disrupted interactions only with talin were resistant to both pulmonary thromboembolism and thrombosis of the carotid artery, but exhibited limited bleeding \((138)\). This study suggested that targeting of $\beta_3$ integrin–talin interactions may have advantages over current $\alpha_{IIb}\beta_3$ antagonists due to a reduced risk of pathological bleeding. In addition, membrane permeable peptides corresponding to specific segments of the cytoplasmic tails of $\alpha_{IIb}$ and $\beta_3$ were shown to inhibit activation of $\alpha_{IIb}\beta_3$ \((139-141)\). However, these intracellular approaches to inhibit $\alpha_{IIb}\beta_3$ are only in the early stage of development.

1.7 Rational and hypothesis

Integrin inside-out signalling increases the affinity of integrins for ECM ligands. Ligated integrins generate outside-in signalling that controls many critical intracellular changes. Both inside-out and outside-in signalling of $\alpha_{IIb}\beta_3$, the
predominant integrin expressed in platelets, is important to form a stable thrombus in vivo. Binding of fibrinogen by the extracellular domain of α\textsubscript{IIb}β\textsubscript{3} directly supports platelet-platelet interactions. Occupied α\textsubscript{IIb}β\textsubscript{3} integrins cluster and trigger outside-in signalling that stabilizes the aggregate and supports wound healing responses.

The interaction of intracellular molecules with the integrin’s cytoplasmic domain regulates the integrin activation state and/or integrin outside-in signalling. There are particular amino acids or motifs in both α and β cytoplasmic tails of α\textsubscript{IIb}β\textsubscript{3} that serve as binding sites for intracellular binding partners. Their interactions could generate an activation signal that leads to conformational changes within the ligand binding site, resulting in the expression of a competent receptor. In addition, some interactions occur after ligand binding, and could influence outside-in signalling and regulate intracellular events, such as cytoskeleton reorganization, cell spreading and motility.

Among the cellular proteins that can directly interact with integrin cytoplasmic domains, skelemin is of particular interest as it can bind with the membrane proximal regions of both α\textsubscript{IIb} and β\textsubscript{3} tails and shows dynamic co-localization with α\textsubscript{IIb}β\textsubscript{3} in newly formed adhesions (96) (108). We hypothesize that skelemin is not involved in the regulation of integrin activation, but is an important modulator for integrin outside-in signalling and acts to fine-tune the cell spreading process together with other cytoskeletal proteins.

In addition, both α\textsubscript{IIb} and α\textsubscript{v} integrin subunits can pair with β\textsubscript{3} subunits to form functional receptors, and they also share the central-turn motif within their cytoplasmic tails. These characteristics reveal a possible convergence in the
regulatory control of αIIbβ3 and αvβ3. Previous studies showed the antagonistic effects of the turn motif peptide derived from αv tails. Here, it is hypothesized that the turn motif of αIIb tails is important for the regulation of integrin functions. The turn motif-derived peptide (αIIb peptide) may not only inhibit αIIbβ3-ligand engagement, but also target αv integrins. To explore this hypothesis, the modes of function of the αIIb peptide have been investigated.

1.8 Specific aims

1.8.1 Aim 1: Evaluate the contribution of skelemin-αIIbβ3 interaction to integrin bi-directional signalling

To determine the functional consequences of these interactions on αIIbβ3 bi-directional signalling, expression of αIIbβ3 on CHO cells was used to examine integrin inside-out and outside-in signalling. A series of stable CHO cell lines were established, which expressed mutant αIIbβ3 receptors where skelemin binding residues at the membrane proximal region of integrin tails were mutated by alanine substitution. Cell surface expression of receptors and integrin activation states of mutant cells were first examined by flow cytometry. Transfection of skelemin immunoglobulin C2 motifs 4-5 that contain the αIIbβ3-binding domain (skeC2) into wild-type and mutant cells was used to characterize whether skelemin is involved in the regulation of integrin activation. To study the effects of the skelemin-integrin interaction on integrin outside-in signalling, three major experimental techniques were used, cell adhesion, cell spreading, and activation of downstream signalling molecules.
Confocal microscopy experiments were employed to determine whether skelemin colocalizes with αIIbβ3 in wild-type and mutant cells. As talin contact sites on αIIbβ3 overlap those of skelemin, the study of skelemin and talin colocalization with αIIbβ3 also gives insights into whether the binding of the two proteins with integrin is mutually exclusive.

1.8.2 Aim 2: Determine the mode of action of bioactive cell permeable cytoplasmic peptides

There are activating and inhibitory functional motifs within the cytoplasmic tails of αIIbβ3. To test the capacity of cytoplasmic peptides to influence integrin activation on live cells, myristoylation of peptides was used as a means of efficient and non-invasive intracellular delivery. Our previous data indicate that the inhibitory capacity of the αv cytoplasmic tail peptide resides near the PP-turn motif. A peptide that derived from the turn motif of αIIb tails, its homologous αv peptide, and mutant peptide RPP/AAA and its scrambled form were synthesized. The functional properties of these peptides were examined in αIIbβ3-expressing CHO cells and the breast cancer cell lines, MDA-MB-435 and MDF-7. Cell adhesion assays and soluble ligand binding were performed to examine the inhibitory capacity and specificity of the peptides. To identify proteins that interact with the αIIb peptide, CHO cell lysates were immunoprecipitated with biotinylated peptides. Bound proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), excised and subjected to mass spectrometry analysis. Furthermore, integrin downstream signalling
molecules in peptide-treated cells were examined with Western-blot. Whether the peptides still possess inhibitory capacity after ligand engagement was also investigated.
CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture

CHO, MDA-MB-435 and MCF-7 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Stable α₁β₃-expressing CHO cells were maintained in the presence of 400 μg/ml G418 (Geneticin) and 300 μg/ml hygromycin B and cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hek293 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

2.2 Generation of stable α₁β₃-expressing CHO cell lines

The cDNAs for α₁β₃ integrin subunits were cloned into pcDNA3.1 vectors harbouring neomycin and hygromycin resistance genes respectively. Mutations were carried out with the use of a site-directed mutagenesis strategy. All sequences were verified by DNA sequencing. CHO cells were transected using Lipofectamine (Invitrogen Corp.) with the respective plasmids for both α₁β₃ subunits according to the manufacturer’s instructions. At 48 hours after transfection, cells were harvested, diluted, and grown in fresh medium containing the selection reagent, G418 (600 μg/ml) and hygromycin B (500 μg/ml). Selection media was changed every 2-3 days.
until cell colonies formed. To obtain single cell clones, cells were treated with Trypsin-EDTA for 3 minutes before cells completely detached. Each colony was gently sucked up with a 200 μl pipette and immediately transferred to a 96-well plate containing the appropriate selection agent. After the cells grew to full confluence, they were harvested and their integrin expression levels were evaluated with flow cytometry. Briefly, 1×10⁶ cells from each clone were trypsinized, suspended in PBS and incubated with 5 μg/mL anti-β₃ antibody (CD61, Invitrogen) for one hour on ice, followed by labelling with goat anti-mouse Alexafluor 488-labelled antibody (Invitrogen), and then analyzed using flow cytometry. The clones with the highest fluorescence intensity were chosen and grown in culture medium containing 400 μg/ml G418 and 300 μg/ml hygromycin B. After 1-2 months growth, a stable cell line was generated with more than 95% β₃ positive.

2.3 Flow cytometry

CHO cells were grown to 70–80% confluence and trypsinized. After quenching with complete medium, the cells were suspended in Tyrodes buffer containing 1 mM CaCl₂, 1 mM MgCl₂, 0.1% glucose and 0.1% BSA. To measure cell surface levels of integrin, CHO cells were incubated with 5 μg/mL anti-β₃ mAb (Invitrogen) for one hour on ice. Cells were then washed, incubated with a secondary goat anti-mouse phycoerythryn (PE)-labelled antibody, and analyzed by flow cytometry.

For PAC-1 binding, 5×10⁵ cells were harvested and pre-treated with Tyrodes buffer containing 2 mM Ca²⁺, 200 μM Mn²⁺ or 2 mM EDTA at 37°C for 15 minutes.
Cells were then incubated with PAC-1 (10 μg/ml, Becton Dickinson) at room temperature for one hour, washed and then incubated with PE-labelled goat anti-mouse Ig (BD Biosciences) for one hour on ice. Cells were analyzed by flow cytometry. The mean fluorescence intensity was used to provide a measure of ligand binding affinity of integrins.

To assess the effect of skelemin fragment expression on the integrin affinity state, cells were transiently transfected with green fluorescence protein (GFP) or GFP-skeC2 using Lipofectamine (as above). After 48 hours, cells were harvested and pre-treated with Tyrodes buffer containing 2 mM Ca\(^{2+}\), 200 μM Mn\(^{2+}\) or 2 mM EDTA at 37°C for 15 minutes. PAC-1 binding assays were carried out following the above method. An integrin activation index was used to compare integrin activation levels in GFP and GFP-skeC2 transfected cells for each mutant. The activation index was calculated using the following formula: 

\[
100 \times \frac{(F_{Ca} - F_{EDTA})}{F_{Mn}},
\]

where \(F_{Ca}\) is the mean fluorescence intensity (MFI) of PAC-1 binding in the presence of Ca\(^{2+}\), \(F_{EDTA}\) is the PAC-1 binding in the presence of EDTA, and \(F_{Mn}\) is the maximal PAC-1 binding in the cells treated with Mn\(^{2+}\).

### 2.4 Cell adhesion assay

The day before performing the cell adhesion assay, 96-well tissue culture plates were coated overnight at 4°C with 50 μl of 20 μg/ml fibrinogen, 5μg/mL vitronectin or heat-denatured 1% bovine serum albumin (BSA). Cells were grown to 70–80% confluence and detached with Trypsin-EDTA. After quenching the trypsin with
complete medium, cells were suspended in PBS. Cell number was counted on a
coulter counter (Beckman Coulter) to obtain $5 \times 10^4$/well. Cells were incubated with
cell permeable, non-fluorescent dye Calcein AM (10 μM Invitrogen) at 37°C for 30
minutes in the dark. Calcein-AM is hydrolyzed by intracellular esterases to the cell
membrane-impermeable green-fluorescent Calcein. Because the esterase activity is
proportion to the number of viable cells, the fluorescence produced is used as a
measure of the cell number. Labelled cells were washed twice and resuspended at $5 \times
10^5$ cells/mL in Tyrode’s buffer containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1% glucose
and 0.1% BSA. The cells were then added to each well ($4 \times 10^5$ cells in 100 μl) and
incubated at 37°C for 30 minutes. Once cells were added, the cell fluorescence
intensity for each well (FL1) was measured on the SpectraMax M2e microplate reader
at 494 nm excitation and 517 nm emission wavelengths. After adhesion on ligand at
37°C for 30 min, unbound or weakly attached cells were removed by washing twice
with PBS. The cell fluorescence intensity after washing (FL2) was measured. Cell
adhesion was quantified with the percentage of fluorescence intensity of attached cells
relative to that of total cells ($100 \times \text{FL2/FL1}$).

For peptide treatment, the peptide stock solution (2.5 mM) was added into cell
suspensions after cells were labelled by Calcein AM. Cells were treated with peptides
for 20 minutes at 37°C. Then 100 μL of cell suspension ($5 \times 10^4$/well), in the presence
of peptides, was added to a ligand-coated 96-well plate. The cell fluorescence
intensity before and after washing was measured and cell adhesion was quantified as
described above.
For function-blocking cell adhesion experiments, labelled breast cancer cells were incubated with 10 μg/mL of each antibody for 20 minutes at room temperature. Then 100 μL of cell suspension (5 × 10⁴/well) in the presence of antibodies was added to a ligand-coated 96-well plate. Three antibodies were used as function-blocking antibodies; αvβ3 (MAB1976, Millipore), αvβ5 (MAB1961, Millipore) and αvβ6 (MAB2077Z, Millipore). The cell fluorescence intensity before and after washing was measured and cell adhesion was quantified as described above.

2.5 Platelet aggregation assay

Blood was drawn by venepuncture using 3.2% buffered sodium citrate as the anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for 15 minutes and used within 4 hours. The PRP was carefully removed and platelet poor plasma (PPP) was obtained by centrifuging the remaining blood at 1200 g for 15 minutes. The platelet count of PRP was measured on a coulter counter (Beckman Coulter) and adjusted to 2 x 10⁸ platelets/ml using PPP. PRP (450 μl) was transferred to siliconized glass aggregometer tubes. The aggregometer tubes were transferred to a 560CA whole blood lumi-aggregometer (Chronolog Corp.) which measures the changes in light optical density for each sample. The PRP was warmed to 37°C for five minutes. In peptide post-treatment assay, platelet aggregation was initiated by the addition of ADP (10 μM) and epinephrine (EPI) (20 μM). Five minutes later, 50 μl of the test peptide or control peptide were added and the samples were monitored for an additional five minutes. In peptide pre-treatment assay, the PRP was treated with 50
μM peptides. Then ADP (10 μM) and EPI (20 μM) were added and platelet aggregation was monitored.

2.6 Immunohistochemistry

Falcon 4-well culture slides were treated with 1% SDS, rinsed with PBS and then coated with 20 g/ml of fibrinogen or 5 μg/ml vitronectin overnight at 4°C. Cells were seeded and adhered to culture slides for different times. Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized by 0.2% (V/V) Triton X-100 for 10 minutes, washed three times and blocked with 1% BSA in PBS. Filamentous actin (F-actin) was stained using Alexa Fluor 594 phalloidin (Invitrogen) for 30 minutes at a 1:40 dilution, and visualized using fluorescence microscope.

Focal adhesions were stained using an antibody to vinculin (hVin-1, Sigma) and then a fluorescein-conjugated secondary antibody. For visualization of integrin distribution, cells were fixed in 4% paraformaldehyde, incubated with β3-specific mAb (AP3, GTI Diagnostics) overnight at 4°C and then a fluorescein-conjugated secondary antibody for two hours. Permeabilization is not required for staining integrin receptors. For visualization of integrin and skelemin/talin colocalization, β3 integrins were first stained as described above, and then talin (C-20, Santa Cruz) or skelemin (rabbit IgG from Dr. T. Ugarova) was stained with BD Cytofix/Cytoperm solution (BD Biosciences) following the manufacturer’s instructions. This sequential approach preserved the cell surface antigens during the intracellular staining.
2.7 Co-immunoprecipitation

Wild-type and mutant CHO cells were transfected with GFP-skeC2 plasmids for 48 hours, then harvested and allowed to adhere to fibrinogen-coated dishes for one hour at 37°C. Cells were solubilized with lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM CaCl₂, 1 mM Phenylmethylsulfonyl Fluoride (PMSF), 100 μg/mL leupeptin, and 10 mM benzamidine] for one hour at 4°C. After centrifugation at 12,000 rpm (15 minutes, 4°C), the supernatant was collected as total cell proteins. Protein concentrations of lysates were determined via the bicinchoninic acid (BCA) assay (Sigma). Lysis buffer was added to make each sample up to a final volume of 600 μl, and contain equal amount of protein for each co-immunoprecipitation (600 μg to 1000 μg total proteins). The lysates were incubated with 2 μl anti-GFP serum (Invitrogen) for two hours at 4°C. The immunocomplexes were captured by incubation with 40 μl of Protein G-agarose overnight at 4°C and washed three times. The immunocomplex samples were boiled in 20 μl 3× sample loading buffer [New England Biolabs; 187.5 mM Tris-HCl, 6% sodium dodecyl sulphate (SDS), 30% glycerol and 0.03% phenol red] for 5 minutes. Samples were then frozen or prepared for Western blot analysis.

2.8 Western-blot

CHO cells were harvested, washed and suspended in Tyrodes buffer containing 1 mM CaCl₂, 1 mM MgCl₂, 0.1% glucose and 0.1% BSA. Cells were allowed to adhere on fibrinogen-coated culture dishes for one hour at 37°C. The buffer was removed,
and cells were scraped from the tissue culture dish in cold PBS. Cell suspension was pooled, centrifuged, and the cell pellet was lysed for one hour at 4°C in 120 μl of lysis buffer (CellLytic™ M, Sigma) with protease inhibitor cocktail (Sigma) and protein phosphatase inhibitor (Millipore).

After starving with FBS-free media for 12 hours, MDA-MB-435 cells were detached with Trypsin-EDTA. Trypsin was quenched with complete medium and cells were then resuspended in Tyrode’s buffer. Cells were incubated with 100 μM peptides at 37°C for 20 minutes, and plated on vitronectin-coated dish. After 1 hour to allow for adhesion at 37°C, floating cells as well as adherent cells were collected and lysed in lysis buffer (CellLytic™ M, Sigma) that contained protease inhibitors (Sigma) and protein phosphatase inhibitors (Millipore).

Cell lysate was kept on ice for 1 hour, followed by centrifugation at 12,000 rpm (15 minutes, 4°C). Protein concentrations of supernatant were determined via BCA assay (Sigma) to make sure of equal protein loading. Total proteins (50-100 ug) were boiled for 5 minutes in 3x sample loading buffer (New England Biolabs, 187.5 mM Tris-HCl, 6% SDS, 30% glycerol and 0.03% phenol red) and resolved by 7.5% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane (Whatman) with a semi-dry protein transfer apparatus (40 minutes at 240 mA). The membrane was blocked with 5% TBST (Tris-Buffered Saline and Tween 20) blocking milk [TBS (100 mM Tris-Cl pH 8, 150 mM NaCl), 0.01% Tween (v/v), 5% non-fat milk] for 30 minutes at room temperature. 5% BSA in TBST was used to block membranes for detecting phosphorylated proteins. After blocking, membranes were incubated
overnight at 4°C with primary antibodies as follows: anti-pY416-Src (100F9, Cell Signalling), anti-Src (mAb327, Calbiochem), anti-β3 antibody (N20, Santa Cruz), anti-pY397 FAK (Santa Cruz), FAK (A-17, Santa Cruz), β-actin (Santa Cruz), phospho-Erk1/2 (197G2, Cell Signalling), and Erk1 (K-23, Santa Cruz).

Primary antibody was removed and membranes were washed 4-5 times with TBST. Membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase in 5% TBST blocking milk for two hours at room temperature. Membranes were then washed with TBST three times. Proteins were detected by rinsing membranes with a 1:1 dilution of Chemiluminescence Substrate (PerkinElmer) and exposing them to X-ray film (Santa Cruz).

2.9 Peptide synthesis

Peptides were synthesized on an Applied Biosystems 433A peptide synthesizer at 0.25 mM scale using fourfold excess of 9-Fluorenylmethyl-carbonyl (Fmoc)-amino acids relative to the p-alcoxybenzyl-alcohol resin (Wang resin). Coupling was performed with 0.5 M N-Hydroxybenzotriazole/O-Benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HOBt/HBTU).

The myristoylated peptides were synthesized by a covalent linkage of myristic acid to the N-terminal amino group of the lysine residue. Briefly, 1 ml N,N-Diisopropylethylamine (DIEA) in dimethylformamide (DMF) was added to the peptide-resin vessel and mixed for 5 minutes. The resin was drained and washed with DMF two times. 520 mg myristic acid was dissolved in dichloromethane (DCM) with
heating (at about 40 °C, 10-15 seconds) and vortexing. Repeat heating and vortexing process until fully dissolved. Then 460 mg N,N'-Dicyclohexylcarbodiimide (DCC) dissolved in DMF were added to the myristic acid solution. After 5 minutes, the mixture of myristic acid and DCC was added to the peptide-resin vessel, with myristoylation was carried out under constant shaking. Myristoylation was monitored by the use of ninhydrin test (Applied Biosystems), which yields deep-blue or purple color by reacting with primary amines when the N-termini of peptides are not myristoylated. Completion of myristoylation was shown by a colorless negative result.

The myristoylated peptides on resin were washed and dried under high vacuum over night. The side-chain protecting groups on peptides were removed and peptides were cleaved from the resin by treatment with 10 ml trifluoroacetic acid (TFA):phenol:water:triisopropylsilane (TIPS) (88:5:5:2) for 4 hours.

Crude peptides were purified by semi-preparative reversed phase high-performance liquid chromatography (HPLC) on a C18 column (C18 is a silica-based column with octadecyl carbon chains bonded on silica). Purified peptides were analyzed on a Varian ProStar HPLC using a C18 column, 250 × 4.6 mm.

The biotin group was labelled on the amino group of Lys side chain. Briefly, 20 mg myristoylated peptide was dissolved in 4 ml DMF. 175 μl DIEA was added to the peptide solution. Immediately before use, a solution of N-Hydroxysuccinidobiotin (NHS-Biotin) was prepared by dissolving 19 mg NHS-Biotin in 500 μl DMF. The NHS-Biotin solution was then added to the peptide solution. The reaction mixture was
protected from light and stirred with a magnetic stirring bar. After 4 hours of reaction, biotinylated peptides were purified by semi-preparative reversed phase HPLC on a C18 column.

The molecular weight of each peptide was confirmed by mass spectrometry and the purity was over 98%, as assessed by analytical HPLC (Figure 2.1A and B). The peptides were dissolved in 10% ethanol with pH 7-9 adjusted with 1M KOH. Cellular uptake and intracellular distribution of bio-peptides was studied by immunocytochemistry. The immunofluorescence microscopic image of the bio-\(\alpha_v\) peptide distribution is representatively shown in Figure 2.1C.

### 2.10 Peptide electrophoresis on 20% SDS-PAGE gel

The electrophoretic mobility of peptides was assessed by using a 20% SDS-PAGE gel. Gels were cast and run using the BioRad Mini-PROTEAN® Electrophoresis system (BioRad). To prepare 6 ml separating gel solution, 3 ml 40% acrylamide, 2 ml 3 M Tris-Cl (pH 8.45), 60 \(\mu\)L 10% SDS(w/v), 200 \(\mu\)L H\(_2\)O and 634 \(\mu\)L glycerol were mixed. 50 \(\mu\)L ammonium persulfate (APS) and 5 \(\mu\)L tetramethylethylenediamine (TEMED) were added, mixed gently and then immediately poured between two glass plates immobilized in a casting apparatus. The gel solution was topped with a thin layer of ethanol to ensure a level surface. To prepare the stacking gel, 0.26 ml 40% acrylamide, 0.62 mL 3 M Tris-Cl (pH 8.45), 20 \(\mu\)L 10% (w/v) SDS and 1.62 mL H\(_2\)O were mixed. Once the separating gel is solidified, the ethanol was poured off and a stacking gel solution was poured on top of the separating gel and a gel comb was
Figure 2.1 Purity and cellular distribution of the αv peptide. (A, B) Histograms show HPLC tracings of the αv (A) and bio-αv (B) peptides. The solvent was aqueous acetonitrile with 0.1% TFA run at a flow rate of 1 ml/min. The gradients were 1–60% acetonitrile over 60 min. Detection was light absorption at 215 nm, where the peptide bond absorbs. (C) Cellular distribution of the bio-αv peptide. The αv peptide was biotin labelled. After treated with bio-αv for 30 minutes, MCF-7 cells were fixed in 4% paraformaldehyde for 10 min, and permeabilized by 0.2% (V/V) Triton X-100 for 5 min. Peptide was then stained by PE-labelled avidin and visualized by immunofluorescence microscope. The picture showed that most peptides were accumulated on the plasma membrane, as indicated by the blue arrow.
inserted in the stacking gel. Before electrophoresis, 1 µg peptide sample was incubated with 3X SDS sample buffer (New England Biolabs; 187.5 mM Tris-HCl, 6% SDS, 30% glycerol and 0.03% phenol red, 0.125 mM DTT) at 70°C for 10 min. The gel was run in tricine-SDS running buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS) at a constant 150 Volts until desired peptides or proteins were sufficiently separated. Peptide bands were visualized by staining with 0.025% (w/v) Coomassie Blue R250. Briefly, the gel was placed in fixative solution (40% methanol, 10% acetic acid) and equilibrated for 30 minutes. Gels were stained in stain solution (0.025% (w/v) Coomassie Blue R250, 10% acetic acid) for one hour and destained in 10% acetic acid three times for 15 minutes or until the desired background was achieved.

2.11 Mn$^{2+}$ stimulated fibrinogen binding assay

$\alpha_{\text{IIb}\beta_3}$-transfected CHO cells were harvested, and suspended at 1×10$^7$/ml in Tyrodes buffer containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1% glucose and 0.1% BSA. 100 µl cell suspension was treated with different concentrations of each peptide for 15 minutes at 37°C, and stimulated with 400 µM Mn$^{2+}$ for another 15 minutes. 5 µl 1.5 mg/ml Alexafluor 488-labelled fibrinogen (Molecular Probes) was then added to the cell suspension and incubated for 45 minutes at room temperature. Cells were washed twice with fibrinogen binding analyzed by flow cytometry.

In addition to flow cytometry, CHO cells prepared as above were analyzed by immunocytochemistry. Cell cultures were fixed with 4% paraformaldehyde for 10 minutes and stained with anti-$\beta_3$ antibody (AP3, GTI. Diagnostics), followed by the
addition of PE-labelled goat anti-mouse antibody. Cells in mounting media were plated on coverslips and visualized by confocal microscopy.

2.12 Co-immunoprecipitation with streptavidin-coated Dynabeads

25 µl Streptavidin-coated Dynabeads (M-280, Invitrogen) were washed in PBS three times, re-suspended and incubated for 2 hours at room temperature with constant rotation with or without 20 µg biotinylated peptides. Beads were washed four times in PBS. αⅡbβ3-transfected CHO cells were harvested and lysed in lysis buffer (CelLytic™, sigma) with protease inhibitor (Sigma). 120 µl cell lysate was diluted with 450 µl PBS, and incubated with activated beads at 4°C overnight. Unbound proteins were removed by extensive washing in a buffer (20 mM tris pH 7.4, 1% triton-100, 50 mM NaCl, 1 mM Ca²⁺, 1 mM Mg²⁺). Associated proteins were eluted by boiling for 8 minutes in SDS sample buffer with DTT. Proteins were separated by 7.5% SDS-PAGE and visualized within the gel using silver staining (ProteoSilver™ Silver Stain Kit, Sigma). The gel bands of interest were cut out, trypsinized and the proteins within them were identified by mass spectrometry.
CHAPTER 3

SKELEMIN IN \( \alpha_{11b}\beta_3 \) –MEDIATED CELL SPREADING

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3.1 Introduction

The role of integrin in cell adhesion, spreading and migration relies on its connection to the cell cytoskeleton. A numbers of proteins are recruited directly or indirectly via adaptors to the cytoplasmic tails of integrin, for example, talin, filamin, \( \alpha \)-actinin and myosin (71). These proteins promote integrin-actin cytoskeleton linkage which influences reorganization of the actin cytoskeleton and generates traction forces necessary for cell spreading and migration. Skelemin was reported to bind to the \( \beta_1 \) and \( \beta_3 \) cytoplasmic tails via yeast two-hybrid assays (142). Skelemin is a cytoskeletal protein first identified in the periphery of the sarcomeric M-line of myosin thick filaments in striated muscles (143). In muscle cells, skelemin cross-linked myosin filaments to maintain the thick filament lattice (144), and to serve as a linker between M-band and intermediate filaments through a desmin binding domain (107). Skelemin is a myomesin isoform and belongs to a member of a family of myosin associated proteins. Skelemin and myomesin are encoded by the same gene but alternative
splicing gives rise to the insertion of a serine/proline-rich domain in the center of skelemin (145). Recent studies have confirmed the presence of skelemin in non-muscle cells, such as platelets and CHO cells (108, 142, 146). In addition, after adhering to immobilized ligand fibrinogen, skelemin can interact and co-localize with integrin αIIbβ3 at the initial stage of cell spreading, suggesting that skelemin serves as a cross-linker between integrin and the myosin cytoskeleton in non-muscle cells (108, 142, 146).

Skelemin is one of very few proteins reported to bind to both the αIIb and β3 cytoplasmic tails of an integrin (96, 146). It contains five repeats of fibronectin type III motifs and seven repeats of immunoglobulin superfamily C2-like motifs (107). The primary interaction of skelemin with αIIbβ3 involves the skelemin immunoglobulin C2 motif 5 and the membrane proximal regions of the αIIbβ3 cytoplasmic tails, while there is an additional low affinity contact between the skelemin immunoglobulin C2 motif 4 and the C-terminus of β3 tails (96, 142). However, the functional significance of skelemin-integrin interactions has not been fully explored. In this chapter, integrin affinity state, outside-in signalling and related functions in CHO cells overexpressing mutant integrins lacking the capacity of bind skelemin were investigated. Dr. Haas and his collaborators previously identified the critical residues in the αIIb and β3 tails involved in skelemin binding (146). Here, alanine substitutions were introduced at Arg995, Arg997 and Leu1000 in the αIIb tail region, and Lys716 and His722 in the β3 tail region (Figure 3.1). Stably expressed single, double or triple mutations in CHO cells were established, namely R995A,
R997A, R995A/R997A, L1000A, R995A/R997A/L1000A, K716A, H722A, and R995A/R997A/K716A. Integrin-mediated cell adhesion, cell spreading, activation of FAK and Src were investigated, and the distribution of $\alpha_{\text{IIb}}\beta_3$, skelemin and talin were measured in the protrusions of the cell leading edge.

### 3.2 Results

#### 3.2.1 Integrin expression

The expression of wild-type and mutant integrin was assessed by flow cytometry using a $\beta_3$-specific antibody (Figure 3.1). The percentage of cells expressing $\alpha_{\text{IIb}}\beta_3$ receptors in the mutant cell lines were comparable to those of wild-type cells, except for K716A which had only 70% of cells expressing comparable amount of integrins.

#### 3.2.2 Association of skelemin with mutant integrins

Experiments were then performed to confirm that our mutations resulted in a decreased association of skelemin with the expressed integrins. In cells, skelemin is present as either a soluble cytoplasmic protein or as an insoluble cytoskeletal-bound protein. The relative abundance of soluble endogenous skelemin in our cell lines was very low and therefore the interaction between endogenous skelemin and $\alpha_{\text{IIb}}\beta_3$ was difficult to evaluate by co-immunoprecipitation and western blot. Therefore, to overcome this problem, co-immunoprecipitation experiments were performed using recombinant GFP-skeC2 fusion protein (skeC2: skelemin immunoglobulin C2 motifs...
Figure 3.1 Amino acid sequences of α_{IIb} and β_{3} cytoplasmic tails and cell-surface expression levels of wild-type and mutant α_{IIb}β_{3} CHO cells. (A). Amino acid sequences of α_{IIb} and β_{3} cytoplasmic tails. Residues targeted for alanine substitutions are underlined and sequence numbers displayed. (B). Cell-surface expression levels of CHO cells transfected with wild-type and mutant α_{IIb}β_{3}. Cells were harvested, and incubated with Tyrode-Hepes Buffer alone (grey line) or with an anti- β_{3} antibody (black line). A PE-labelled secondary antibody was used to detect bound anti-β_{3} antibody and flow cytometry analysis was performed in Tyrode-Hepes buffer. Cells incubated only with the secondary antibody (grey line) were used as controls. Histograms were generated using FlowJo. The data are representative of three separate experiments.
4-5 that contain the $\alpha_{IIb}\beta_3$-binding domain). Wild-type, R995A/R997A/L1000A, H722A and K716A cells were transiently transfected with plasmids of GFP-skeC2 or GFP alone (control). After 48 hours of transfection, cells were harvested and allowed to adhere to immobilized fibrinogen for 1 hour. Cell lysates were subjected to immunoprecipitation with antibodies against GFP, then western blot analysis (Figure 3.2). Two GFP-immunoreactive proteins were detected, GFP ($\sim 27$ kD) and the GFP-skeC2 fusion protein ($\sim 90$ kD). An apparent MW of 90 kD is consistent with the predicted size of the fusion protein. A long transfer time (40 min) was used to ensure sufficient transfer of proteins from the gel to the nitrocellulose membrane under semi-dry transfer conditions. Low MW proteins in a pre-stained protein ladder passed through the membrane, which indicated that some GFP proteins ($\sim 27$ kD) might be also over-transferred, leading to decreased band intensity (Figure 3.2). Immunoblotting with an anti-$\beta_3$ antibody revealed that $\beta_3$ proteins were co-immunoprecipitated with GFP-skeC2, but not with GFP (Figure 3.2). In comparison to wild-type cells, there appeared to be a decrease in the amount of $\alpha_{IIb}\beta_3$ co-immunoprecipitated with R995A/R997A/L1000A, H722A and K716A mutated integrins. Thus, we confirmed that the mutations did result in a decrease association of skelemin with $\alpha_{IIb}\beta_3$.

### 3.2.3 Integrin affinity for ligands

The membrane proximal regions in the integrin cytoplasmic domains are important for integrin activation modulation, and point mutations within this region
Figure 3.2 Association of GFP-skeC2 with αIIbβ3. Wild-type, R995A/R997A/L1000A, H722A and K716A mutant cells were transfected with GFP-skeC2 plasmids for 48 hour, and then allowed to adhere to immobilized fibrinogen for one hour. Cell lysates were immunoprecipitated with anti-GFP. Western blot analysis using anti-β3 antibody revealed a decreased association between GFP-skeC2 and the mutant αIIbβ3 receptors compared to wild-type αIIbβ3.
could enhance the affinity for integrin ligands and promote constitutive signalling (39, 147). To assess the activation state of αIIbβ3, flow cytometry was used to study αIIbβ3 binding to PAC-1, which recognizes the conformationally active form of αIIbβ3 (Figure 3.3). αIIbβ3 binding to its ligand requires divalent cations (Ca$^{2+}$ or Mg$^{2+}$). Optimal αIIbβ3 binding to ligands occurs in the presence of Ca$^{2+}$ at a concentration of 1-2 mM, a physiological Ca$^{2+}$ concentration in human plasma (148). In the current study, 2 mM Ca$^{2+}$ was used. EDTA chelates either Ca$^{2+}$ or Mg$^{2+}$ and is used as an inhibitor of integrin ligand binding. PAC-1 binding in the presence of EDTA represented non-specific ligand binding, which was subtracted from the total binding (in the presence of divalent cations) to yield specific binding. Low and negligible PAC-1 binding was observed in the presence of EDTA, suggesting that Ca$^{2+}$-mediated PAC-1 binding was specific (Figure 3.3C). In the presence of Ca$^{2+}$, wild-type αIIbβ3 expressed in CHO cells was in the resting state, while R995A, K716A and R995A/R997A/L1000A cells bound significant levels of PAC-1, showing that the three mutants are active (Figure 3.3A). These data are consistent with previous mutational studies (39, 149), suggesting that the residues K716 and R995 within β3 tails are involved in the regulation of αIIbβ3 activation.

Mn$^{2+}$ has a higher affinity for integrins than Ca$^{2+}$ or Mg$^{2+}$. It strikingly increases the ligand binding affinity of almost all integrins by binding to integrins and inducing conformational changes of the integrin ectodomain from a bent to an extended and active state (150). Therefore, Mn$^{2+}$ has been widely used as a positive control for integrin activation. PAC-1 binding under the condition of Mn$^{2+}$ treatment
Figure 3.3 PAC-1 binding in the presence of metal ions. (A) PAC-1 binding in the presence of Ca\(^{2+}\). Cells treated with 2 mM Ca\(^{2+}\) or 2 mM EDTA were incubated with PAC-1, washed, incubated with a PE-conjugated secondary antibody, and analyzed by flow cytometry. Binding was expressed as mean fluorescence intensity (MFI) of PAC-1 staining in the presence of Ca\(^{2+}\) minus that obtained in the presence of EDTA. The data represent the mean ± S.E. of three separate experiments. (B) PAC-1 binding in the presence of Mn\(^{2+}\). (C) PAC-1 binding in the presence of EDTA. Binding was expressed as MFI of PAC-1 staining in the presence of 200 μM Mn\(^{2+}\). The data represent the mean ± S.E. of two separate experiments.

was also tested (Figure 3.3B). Generally, Mn\(^{2+}\) was able to activate wild-type and mutant α\(\text{IIb}\)β\(3\) leading to a much higher level of PAC-1 binding. However, Mn\(^{2+}\) did not maximally activate α\(\text{IIb}\)β\(3\) integrin as Mn\(^{2+}\)-induced PAC-1 binding in wild-type cells was still significantly lower than that of K716A cells treated with Ca\(^{2+}\) alone. Even though K716A and R995A/R997A/L1000A were in a activation state, Mn\(^{2+}\) had an additional activating effect for these mutants and enhanced PAC-1 binding 3-4 fold compared to wild-type α\(\text{IIb}\)β\(3\). These results suggest that amongst all the mutants, R995A, K716A and R995A/R997A/L1000A exhibited partial activation in the presence of Ca\(^{2+}\), but that in the presence of Mn\(^{2+}\) K716A and R995A/R997A/L1000A had higher maximal activation than that of wild-type α\(\text{IIb}\)β\(3\).

3.2.4 Transfection with skeC2 did not affect the integrin affinity states

To assess whether skelemin binding is responsible for activation of α\(\text{IIb}\)β\(3\), effects of exogenous skelemin overexpression on integrin activation were investigated by two-color flow cytometry (Figure 3.4). In this assay, wild-type and mutant cells were transiently transfected with either GFP-tagged skeC2 or GFP alone. Cells were harvested 48 hours after transfection, incubated with PAC-1 and then stained with
Figure 3.4 SkeC2 did not change integrin activation states in GFP-skeC2 transfected wild-type or mutant cells. Cells were transiently transfected with vectors encoding GFP or GFP-skeC2. After 48 h, cells were harvested and analyzed by two-color flow cytometry for binding of PAC-1 binding. Integrin activation index was used to compare integrin activation levels in GFP and GFP-skeC2 transfected cells for each mutant. The activation index was calculated using the formula described in Materials and Methods. The data represent the mean activation index ± S.E. of three separate experiments. The difference of activation index in GFP or GFP-skeC2 transfected cells was not statistically significant for any of the skelemin mutants using one-way ANOVA ($P<$0.05).
PE-labelled secondary antibody. To study successfully transfected cells, GFP-positive cells were gated for further analysis of PE staining using flow cytometry. The percentage of PE-positive cells in the population of GFP-positive cells was assigned as the PAC-1 binding level in each sample. To study the effect of skeC2 transfection on integrin activation, PAC-1 binding levels in the presence of Ca\(^{2+}\), EDTA or Mn\(^{2+}\) were studied. Therefore, integrin activation can be quantified as an activation index, \(100 \times (F_{Ca} - F_{EDTA})/F_{Mn}\), where \(F_{Ca}\) is the MFI of PAC-1 binding in the presence of Ca\(^{2+}\), \(F_{EDTA}\) is the PAC-1 binding in the presence of EDTA, and \(F_{Mn}\) is the maximal PAC-1 binding in the cells treated with Mn\(^{2+}\). As shown in Figure 3.4, cells expressing either wild-type \(\alpha_{IIb}\beta_3\) or the mutants, R995A/R997A and H722A, GFP-SkeC2 expression did not enhance PAC-1 binding. Similar results were also obtained with the three active mutants, R995A, K716A and R995A/R997A/L1000A. For each version of \(\alpha_{IIb}\beta_3\) integrin, no statistically significant differences were found between cells expressing GFP-SkeC2 and GFP in their ability to bind PAC-1 \((P<0.05)\). Thus, skelemin expression did not appear to alter the affinity state of \(\alpha_{IIb}\beta_3\), suggesting that skelemin is not involved in \(\alpha_{IIb}\beta_3\) activation.

### 3.2.5 Adhesion to immobilized fibrinogen

The strength of cell adhesion to ECM not only depends on integrin expression level and integrin affinity, but also relies on integrin-mediated cytoskeleton linkages. Previous studies demonstrated that skelemin and integrin association was an early response to integrin occupancy and clustering, being initiated between 30 minutes to
two hours after cell adherence. If their association is essential for linking ECM to the cell cytoskeleton, mutant cells may show decreased cell adhesion to ECM. Therefore we compared the capacity of the wild-type cells and cells expressing mutant integrins to adhere to fibrinogen over a 30 minute time period (Figure 3.5). Cells were labelled with fluorescent dye Calcein AM so that the fluorescence intensity of cells was directly proportion to the number of viable cells in each treatment. Fluorescently labelled cells were allowed to adhere to fibrinogen-coated microplates. After washing, adherent cells were counted as a percent of the number of total cells added. Non-specific cell adhesion was low, as less than 5% cells adhered to BSA-coated wells for all cell lines tested. Cell adhesion to fibrinogen was α\textsubscript{IIb}β\textsubscript{3}–mediated, as adhesion of wild-type cells was 3 fold higher than that of mock-transfected cells. As shown in Figure 3.5, cell attachment of R995A, R995A/R997A, R995A/R997A/L1000A, K716A, H722A, and R995A/R997A/K716A to fibrinogen was higher than that of wild-type cells, while R997A and L1000A cell adhesion were comparable. The R995A and K716A mutations showed the strongest cell adhesion, in agreement with their active state as assessed by PAC-1 binding. In general it appears that disruption of the skelemin binding sites in α\textsubscript{IIb}β\textsubscript{3} did not impair stable cell adhesion, suggesting that skelemin-α\textsubscript{IIb}β\textsubscript{3} interaction may not be essential for the linkage of integrin to the cell cytoskeleton.

3.2.6 Cell spreading and membrane protrusions

Integrins and their associated proteins form focal adhesions in cultured cells,
which link integrin clusters to the actin cytoskeleton and initiate actin assembly into stress fibers. Here, stress fibers were stained with fluorescence-labelled phalloidin after cell adhesion to fibrinogen-coated coverslides for one hour (Figure 3.6). Normal CHO cells lacking αIIbβ3 expression were still round at this time point, whereas wild-type and mutant αIIbβ3 transfected cells were spreading and already displaying

![Graph showing cell adhesion results](image)

**Figure 3.5 Adhesion of mutant cells to immobilized fibrinogen.** Calcein-AM labelled cells were plated on 20 μg/ml fibrinogen coated wells and total cell fluorescence were measured on a fluorescence microplate reader. After 30 minutes adhesion in Tyrode’s buffer, weakly and non-adherent cells were washed away and the fluorescence of remaining cells was measured. Cell adhesion, the percent of adherent cells to total cells added, was calculated using the formula described in Materials and Methods section 2.4. Cell adhesion to BSA-coated wells was subtracted as background. The data represent the mean ± S.D. of three separate experiments. **P<0.01 compared to wild-type cells using student’s t-test.
Figure 3.6 Effect of αIIbβ3 mutations on the actin cytoskeleton. (A) After spreading on fibrinogen-coated wells for one hour, cells were fixed, permeabilized and stained with rhodamine phalloidin to detect F-actin. a: CHO; b: wild-type; c: R995A; d: R997A; e: R995A/R997A; f: R995A/R997A/L1000A; g: K716A; h: H722A; and i: R995A/R997A/K716A. Scale bar: 10 μm. (B) Quantitative analysis of cell spreading. The area of each cell was measured using ImageJ analysis software. The mean area of wild-type cells was normalized to 1. Error bars are standard deviations. *P<0.05 compared to wild-type cells using student’s t-test.
strong formation of stress fibers. There was also obvious formation of lamellipodia and filopodia within both wild-type and mutant cell lines. Quantitative measurements of cell areas clearly showed that mutant cells R995A/R997A/L1000A, K716A, H722A and R995A/R997A/K716A exhibited a greater extent of cell spreading (Figure 3.6B). We also stained cells with anti-β3 antibody (AP3) and observed formation of many αIIbβ3-based focal adhesions, lamellipodia and filopodia at the leading edges of the mutant cell lines (data not shown). Taken together, these results demonstrate that defective binding of skelemin to αIIbβ3 does not disrupt actin cytoskeleton organization, or membrane protrusion formation and cell spreading, suggesting that other cytoskeletal proteins are capable of performing some of the functions performed by skelemin. Thus, different cytoskeletal proteins may act in unison to maintain integrin-cytoskeleton linkages.

3.2.7 Src and FAK activation downstream of integrin signalling

The increased cell adhesion ability and higher spreading level observed in some mutations suggested that there was an upregulation in integrin-downstream signalling. Therefore, activation of FAK and Src, two important tyrosine kinases in integrin signalling that are required for efficient adhesion and spreading of cells on integrin ligands were examined. After cell adherence to fibrinogen in Tyrodes buffer for one hour, activation of Src and FAK were assessed by probing the phosphorylation levels of pY416-Src, the positive regulatory autophosphorylation site of Src (151), and pY397-FAK, the major site of tyrosine phosphorylation on FAK (152). Western blot
Figure 3.7 Effect of wild-type and mutant αIIbβ3 on Src and FAK signalling. Cells were allowed to spread on fibrinogen-coated plates for one hour, lysed and total cell extracts were subjected to SDS-PAGE. Blots were probed using antibodies against pY416-Src, Src, pY397-FAK, β3-integrin, GAPDH and β-actin. (A) Analysis of pY416-Src and pY397-FAK in total cell lysates was done on wild-type and mutant cells. (B) Analysis of pY416-Src in total cell lysates was done on wild-type αIIbβ3-expressing cells and mock-transfected cells.
Figure 3.8 Comparable or increased pY416-Src levels in mutant αmβ3-transfected cells. Western blot analysis of pY418-Src in wild-type and mutant cells was repeated in three independent experiments. The graph shows densitometric quantification of pY416-Src/β-actin. Band density was measured by Quantity One software and normalized to the β-actin protein level. The ratio of pY416-Src/β-actin in wild-type cells was assigned as 1. K716A and H722A cells showed significantly increased pY416-Src/β-actin levels compared with wild-type cells using student’s t-test, but the Kruskal–Wallis test revealed no significant difference between mutant and wild-type cells (P<0.05).
analysis of pY416-Src and pY397-FAK showed that comparable or potential higher levels of pY416-Src were observed in mutant cells compared to wild-type cells. However, there were no differences in cellular levels of pY397-FAK between mutant and wild-type αIIbβ3-expressing cells (Figure 3.7A).

To study the effects of αIIbβ3-expression on Src activation, pY416-Src levels in wild-type αIIbβ3 and mock transfected cells were examined. As shown in Figure 3.7B, wild-type αIIbβ3-transfected cells had increased levels of the active form of Src (pY416-Src) than mock-transfected cells when they were plated on fibrinogen. These results suggested that the transfected αIIbβ3 integrins and their binding to ligands attribute to elevated levels of pY416-Src.

Western blot analysis of pY416-Src in different mutant cells was repeated and the blots were quantified as shown in Figure 3.8. Bands were quantified by Quantity One software and normalized to the β-actin protein level. The levels of pY416-Src in K716A and H722A cells were slightly elevated compared with wild-type cells, which were in good agreement with their increased cell spreading.

### 3.2.8 Effect of overexpression of skeC2 on cell spreading

Previous studies have demonstrated that introduction of skeC2 into wild-type cells caused spread cells to round up (108, 142). The similar experiment was performed in Hek293 cells. Hek293 cells were transiently transfected with αIIbβ3, GFP-skeC2 or GFP individually and in combination, and harvested 48 hour after transfection. Transfected cells were allowed to adhere on fibrinogen-coated slides for two hours, then fixed and stained with anti-β3 antibody. As shown in Figure 3.9, the
Figure 3.9 Hek293 cells co-transfected with α_{IIb}β_{3} and GFP-SkeC2 show inhibited cell spreading on fibrinogen. Subconfluent Hek293 cells were transfected with α_{IIb}β_{3}, GFP-skeC2 or GFP individually and in combination, and harvested 48 hour after transfection. Cells were allowed to adhere for 2 hours on fibrinogen-coated slides, then fixed and stained with anti-β_{3} antibody and PE-labelled secondary antibody. GFP and α_{IIb}β_{3} expression were observed with confocal microscopy. Scale bar: 50 μm.
transfection of Hek293 cells with GFP-skeC2 inhibited \( \alpha_{IIb}\beta_3 \)-mediated cell spreading. However, the inhibitory effect only occurred when the expression amount of GFP-skeC2 was relative high.

The effect of overexpression of skeC2 on spreading in mutant \( \alpha_{IIb}\beta_3 \)-transfected CHO cells was also investigated (Figure 3.10). Wild-type, R995A, R995A/R997A/L1000A, H722A and K716A cells transiently transfected with GFP-skeC2 or GFP were re-plated on fibrinogen, and observed by GFP immunofluorescence two hours after spreading (Figure 3.10). Wild-type cells transfected with GFP-skeC2 failed to spread on fibrinogen compared to transfection with GFP alone. A similar result was obtained in R995A cells. However, GFP-skeC2 transfection had less effect on the spreading of R995A/R997A/L1000A, H722A and K716A cells. Notably, R995A/R997A/L1000A, H722A and K716A cells with strong GFP-skeC2 expression (indicated as increased GFP fluorescence intensity) were still able to spread, suggesting that skeC2 expression had little inhibitory effect on cell spreading in these three mutant cell lines.

Figure 3.11 shows a representative confocal microscopic image of transfected wild-type and R995A/R997A/L1000A cells that were stained with anti-\( \beta_3 \) antibody (red) after two hours of spreading. Most wild-type cells expressing GFP-skeC2 were completely rounded up, whereas R995A/R997A/L1000A cells transfected with GFP-skeC2 were fully spread. It was noted that wild-type cells with increased expression of GFP-skeC2 were rounded up but cells that were weakly expressing GFP-skeC2 had normal spreading morphology (indicated by arrow).
Figure 3.10 Immunofluorescence images reveal different effects of skeC2 transfection on wild-type and mutant cells. Wild-type, R995A, R995A/R997A/L1000A, H722A and K716A mutant cells were transiently transfected with GFP-skeC2 or GFP alone, plated on fibrinogen for two hours, fixed, and GFP fluorescence was visualized. Scale bar: 50 μm.
Figure 3.11 Confocal microscopy image of wild-type and R995A/R997A/L1000A cells transfected with GFP-skeC2. Wild-type and R995A/R997A/L1000A cells were transiently transfected with GFP-skeC2 or GFP alone and re-plated on fibrinogen for two hours. Cells were fixed and stained for integrins with AP3 antibody. Integrin distribution (red) and GFP fluorescence were visualized by confocal microscopy. A wild-type cell that weakly expresses GFP-skeC2 is indicated by an arrow. Scale bar: 50 μm.
The above results indicate that skeC2 expression could abolish cell spreading and was more effective in wild-type cells than in mutant cells, at least in R995A/R997A/L1000A, H722A and K716A cells. We conclude from these results that transfected GFP-skeC2 competes with endogenous skelemin for integrin binding, thus disrupting the binding of integrin tails to not only endogenous skelemin, but to other cytoskeletal proteins that physically link $\alpha_{IIb}\beta_3$ to the cell cytoskeleton.

3.2.9 Co-localization of talin and skelemin with integrin $\alpha_{IIb}\beta_3$ in wild-type and K716A mutant cells

Among the other integrin-cytoskeleton linkage candidates, talin is similar to skelemin in that it also has a membrane-proximal binding region in the $\beta_3$ tail, and it also has a distinct NPLY membrane-distal binding site (56). In addition, talin plays critical roles in linking integrin to the actin cytoskeleton and supporting formation of cell membrane protrusions during cell spreading (99, 153). Therefore, double labelling of integrin $\alpha_{IIb}\beta_3$ (red) and talin (green) or skelemin (green) was used to compare the distribution of talin and skelemin in wild-type and mutant cells at an early time point of cell spreading (40 min). Among all the mutant cell lines, K716A was chosen to be shown here (Figure 3.12), as it developed enormous integrin-based membrane protrusions of filopodia and lamellipodia during early cell spreading.

Immunocytochemistry for $\beta_3$-integrin (red) showed that cell attachment to fibrinogen promoted translocation of integrin from the cytosol to the cell periphery in wild-type cells as well as in K716A mutant cells. The K716A mutant cells had a more
Figure 3.12 Distribution of skelemin and talin in wild-type and K716A cells. After spreading on fibrinogen-coated wells for 40 minutes, (A) wild-type or K716A cells were fixed and stained for $\beta_3$ integrin and skelemin or (B) $\beta_3$ integrin and talin. Images were captured using a confocal fluorescence microscope. Scale bar: 50 $\mu$m.
profound degree of spreading level, and filopodia and lamellipodia structures were strikingly obvious. Co-staining for skelemin showed that skelemin colocalized with \( \alpha_{IIb}\beta_3 \) at the cell periphery in wild-type cells, while in K716A mutant cells it was not present in sheets of membrane protrusions at the cell periphery but was found to be localized diffusely in the main cell body, thus losing its co-localization with \( \alpha_{IIb}\beta_3 \) (Figure 3.12A). However, both of the cell lines exhibited marked co-localization of \( \alpha_{IIb}\beta_3 \) and talin at the cell periphery (Figure 3.12B). Wild-type cells displayed very strong ring-shaped staining for both \( \alpha_{IIb}\beta_3 \) and talin at the cell periphery. This strong co-localization pattern is also clearly visible in the sheets of lamellipodia in K716A mutant cells. It seems that co-localization of talin and integrin \( \alpha_{IIb}\beta_3 \) in the regions of cell membrane protrusions in cells transfected with the K716A mutation could be a result of knocking down skelemin binding to \( \alpha_{IIb}\beta_3 \) cytoplasmic tails.

3.3 Discussion

The association of integrins with cytoskeletal proteins is crucial for the transmission of biochemical signals and mechanical force across these adhesion receptors and, thus, for integrin-mediated cell functions, such as spreading, migration and gene expression (154). The dynamic binding of skelemin to the cytoplasmic domains of integrin \( \alpha_{IIb}\beta_3 \) during the cell spreading process has been reported: skelemin did not bind to resting \( \alpha_{IIb}\beta_3 \) in non-adhered platelets and CHO cells; cell adhesion and spreading to immobilized fibrinogen promoted skelemin binding with \( \alpha_{IIb}\beta_3 \); and, the two proteins dissociate in later stages of cell spreading (108, 142, 146).
Since skelemin is a family member of myosin-associated myomesin, it was previously speculated to exert a contractile force by linking integrin to myosin \(142\). In the present study we tested this hypothesis and tried to elucidate the role of skelemin in integrin functions with the use of a series of stable CHO cell lines expressing mutant \(\alpha_{IIb}\beta_3\) integrins in which key residues involved in the binding of skelemin to \(\alpha_{IIb}\beta_3\) were mutated. We recognize that these mutations might also exhibit impaired interactions with other integrin binding proteins, and therefore we generated a number of mutants to reveal a general picture of the functional role of skelemin-integrin interactions. We found that most of the mutant cells defective in skelemin binding had unimpaired cell adhesion and spreading capacity at the early stages of cell spreading on immobilized fibrinogen. Some of the mutant cells also had increased membrane protrusion formation, a larger cell spreading area and elevated levels of activated pY416-Src. These data lead us to conclude that engagement of skelemin to the cytoplasmic tail of \(\alpha_{IIb}\beta_3\) is not essential for the expansion of a cell protrusion during cell spreading. Instead, we propose that the binding of skelemin, talin and other proteins to the tail of \(\alpha_{IIb}\beta_3\) are mutually exclusive events and depending on what protein is bound, a cell will either spread or contract. As discussed below, when skelemin is bound to the cytoplasmic tail of \(\alpha_{IIb}\beta_3\), a contractile force is generated which supports cell contraction. To initiate cell spreading, a cell must prevent or disrupt skelemin binding to allow for the recruitment of other proteins to integrin clusters that facilitate cell spreading, such as talin and Src. Thus, modulating skelemin binding to integrin tails is one mechanism a cell can use to regulate the
highly organized process of cell spreading.

Mutations of R995A and K716A in this study are shown to activate αIIbβ3. To address whether the activation of the mutant is caused by disruption of skelemin binding, we characterized the binding of PAC-1, an antibody specific for activated αIIbβ3 integrin, to cells overexpressing GFP-skeC2. Overexpression of this fragment did not alter integrin affinity state of either wild-type or active mutant cells (Figure 3.4). Unlike talin, which is able to unclasp the membrane-proximal interface of αIIbβ3 cytoplasmic tails and lead to integrin activation, skelemin cannot unclasp the interface even though skelemin also binds the membrane proximal regions of αIIbβ3 cytoplasmic tails. It has been demonstrated that skelemin and αIIbβ3 association occurs after unclasping of the αIIbβ3 interface due to integrin-ligand ligation, which unmasked binding residues recognized by skelemin (146). Our data here support the view that the association of skelemin with αIIbβ3 is a post-ligand-binding event, but not involved in the process of integrin-ligand affinity regulation.

The binding of skelemin with integrin is dynamically regulated during cell spreading, suggesting a regulatory role for skelemin in cell spreading. Our proposed model for skelemin in cell spreading and anchorage is displayed in Figure 3.12. Focusing on cytoskeletal proteins, activation of integrins involves talin binding (Figure 3.12A) that results in integrin clustering and activation of FAK, resulting in the recruitment of skelemin and other cytoskeletal proteins to integrin clusters (Figure 3.12B). During these early phases of cell adhesion and spreading, skelemin competes with other cytoskeletal proteins for binding to the β3 tail and prevents the activation of
Figure 3.13 Proposed model of skelemin interaction with αIIβ3 tails. (A) Activation. Integrin activation involves the binding of talin (T) to the membrane proximal and NPLY regions of β3 that results in unclasp of the integrin tails, leading to αIIβ3 binding to fibrinogen (Fg). Src is also directly bound to the C-terminus of the β3 tail. (B) Early stages of cell anchorage and spreading. Following integrin activation, integrin clustering is initiated together with the activation of FAK (F). This results in the recruitment of additional integrins, skelemin (Sk), and other cytosolic and cytoskeletal proteins to the integrin cluster, and the formation of a focal adhesion. (i) Skelemin binds to the membrane-proximal regions of αII and β3 tails and to the C terminus of β3 tails, which displaces Src from the C-terminus of β3, but still allows Src to bind to β3 via activated FAK bound to the NPLY region. (ii) In the presence of skelemin, talin can still bind to the β3 NPLY region but not to its β3 membrane-proximal binding domain. (iii) The binding of other actin-binding proteins (ABP) to integrin tails allows for maximal Src activation while providing a linkage to the actin cytoskeleton. Skelemin can also provide transient linkages to the actin cytoskeleton (i and ii). A combination of these binding scenarios would be present within a focal adhesion. (C) Late stages of cell anchorage and spreading. As cell adhesion and spreading progresses the majority of the soluble skelemin is replaced by other actin-binding proteins, allowing for increased Src activation. The remaining skelemin makes firm contacts with the actin cytoskeleton, bringing cell spreading and adhesion to completion.
Src from occurring at the C-terminus of the $\beta_3$ tail. In doing so, skelemin would counteract the activity of other proteins involved in cell adhesion, spreading and signalling, thereby providing the cell with mechanism to fine-tune its cell shape. During later stages of cell spreading, soluble skelemin either converts into insoluble skelemin, or dissociates from integrins (146), being replaced by talin or other actin-binding proteins, and firm adhesion occurs (Figure 3.12C).

Previous studies reported that microinjection or over-expression of skeC2 fragments into cultured cells abolished cell spreading (108, 142). It was assumed that skelemin was essential for cell spreading based on the interpretation that skeC2 competes with the binding of endogenous skelemin to integrins but, lacks a cytoskeletal binding site, thus resulting in breakage of the integrin-cytoskeleton linkage. However, we found that mutant cells defective in skelemin binding had unimpaired adhesion and spreading capacity on integrin ligands, suggesting that skelemin binding to integrin was not essential for cell spreading. These apparently contradictory results can be resolved if one reinterprets the earlier study with the new data generated herein. We propose that the binding of skeC2 with integrin tails results in a loss of the control mechanisms governing the dynamics of cell adhesion and spreading by blocking the binding of endogenous skelemin and other cytoplasmic proteins to integrin tails that are important in regulating the formation of cellular protrusions and cell spreading. We found that the use of cells expressing receptors containing site-specific point mutations impeded the binding of endogenous skelemin (but not other cellular proteins) with the integrin tails.
The stoichiometry of skelemin and integrin needs to be considered when using GFP-skeC2 since we found that GFP-skeC2 inhibited the $\alpha_{IIb}\beta_3$-mediated cell spreading only when the proportion of GFP-skeC2 to $\alpha_{IIb}\beta_3$ was high (Figures 3.9 and 3.11). The interaction between integrin and skelemin is robust. A previous NMR study showed that the association between skelemin C2 and integrin $\beta_3$ tail is increased by increasing the ratio of skelemin C2 to $\beta_3$ (II). If the ratio of skelemin C2 to integrin is much higher in plasmid-transfected CHO cells than in wild type cells, such as platelets, one may over-estimate or incorrectly define the role of skelemin in integrin biology. One problem with over-expressing skelemin C2 is that it would saturate the skelemin binding sites on integrins and not only block endogenous skelemin binding but also the association of other integrin binding partners that compete for the skelemin binding site. This is likely why we were able to obtain some novel insights into skelemin function from experiments utilizing our wild-type and mutant integrin cell lines, but not from skelemin C2 over-expression experiments.

The cytoplasmic tails of $\alpha_{IIb}\beta_3$ are key structures for outside-in signalling in that they recruit a substantial number of cell signalling and cytoskeletal proteins (71). Notable amongst these are Src and talin, which are obligatory for cell spreading (153, 155). Data in the current study show that the levels of pY416-Src in H722A and K716A cells were increased (Figure 3.7 and 3.8). Several mechanisms are brought into play following Src activation (151). One is the direct association of the C-terminal region of $\beta_3$ tails with Src, which destroys the autoinhibitory state of Src upon cell adhesion, and integrin clustering stabilizes the activated Src by inducing
intermolecular autophosphorylation (115). We suggest that a reduction in skelemin bound to integrins may allow more Src to be bound and priming it for activation. An NMR spectroscopy study revealed that immunoglobulin C2-like repeat 4 (SkIgC4), interacts weakly with the C termini of β3 tails, which is also the binding site for Src involved in binding with integrin αIIbβ3 (96, 115). Therefore, it is possible that the binding of SkIgC4 with the C termini of β3 tails occupies the Src binding site and thereby reduces the capacity to maximally activate Src. However, it does not completely block Src activation as Src can still bind to and become activated at the membrane distal NPLY motif of β3 through activated FAK (156). Other integrin-associated proteins may play a role in upregulating pY416-Src, such as FAK, PTP SHP-2, and CSK (c-Src kinase), which has been demonstrated to participate in regulating Src activity (113, 152, 157). Mutants in the cytoplasmic tails may also affect the association of integrins with other modulators of Src activation.

Recent structural studies identified the critical roles of K716 of β3 integrin in the αIIbβ3 interface and its interactions with αIIb via hydrogen bonds and electrostatic interactions (149, 158, 159). Our PAC-1 binding assay confirmed that K716A is an activated integrin mutant. Highly developed filopodia and lamellipodia were visualized at the early cell spreading stage in K716A mutant cells (Figure 3.9). Furthermore, the K716 residue appeared to be the most important for skelemin binding in in vitro studies (146). Given the overlapping binding areas within the β3 membrane proximal region, we predict that binding of skelemin and talin to the β3 membrane proximal region are mutually exclusive events. This is supported by our
findings that talin is strongly co-localized with αIIβ3 in the ruffle structure of the K716A cell protrusions, but that skelemin still remained in the main cell bodies (Figure 3.9). Disrupting skelemin binding in K716A mutant cells may facilitate talin recruitment that promotes actin polymerization and membrane protrusion formation.

Filopodia and lamellipodia are the two integrin and actin-based membrane protrusions formed at the leading edge of a moving cell or the periphery of a spreading cell that are prerequisite for cell motility and spreading (6). The elongation of these protrusions pushes the leading edge forward while the tail edge undergoes retraction enabling the cell to migrate (160). Dynamic cell spreading requires a balance of extending and contractile forces. In platelets, contractile forces also play an important role in blood clot retraction, where the fibrin meshwork is bound to αIIβ3 and pulled together by the platelet cytoskeleton. Contractile forces are provided by myosin II (79). Similar to myomesin, skelemin was thought to regulate the organization of myosin filaments and mediate the interaction of myosin with integrins (142). In our study, skelemin was not present in the sheets of lamellipodia, and reducing skelemin-integrin interactions promoted cell protrusion formation in K716A cell and cell spreading in other mutant cells. These results do not support a role for skelemin in generating an extending force but are consistent with skelemin exerting a contractile force.

In summary, our results extend the current understanding of skelemin function as an integrin-cytoskeleton linker. We propose a model in which soluble and insoluble forms of skelemin might differ in their importance for particular integrin functions.
During the initial stages of cell spreading, soluble skelemin proteins bind to $\alpha_{\text{IIb}}\beta_3$ integrin clusters at the leading edges of cells. These skelemin-integrin interactions function to coordinate the binding of different cytoskeletal proteins to the membrane proximal region of integrin tails, such as talin and Src (Figure 3.12B). However, the NPLY region remains exposed during skelemin binding and thus talin and Src can still bind to $\beta_3$. During this time period, talin can therefore function as a linker of integrin and actin filaments, and maximal Src activation can be modulated by skelemin. This might afford a mechanism to dampen Src activation and consequently suppress integrin signalling. As cell adhesion and spreading progresses and large amounts of cell protrusion form, a majority of skelemin then dissociates from integrins to allow for other actin-binding proteins to bind to integrins, bringing cell spreading and adhesion to completion (Figure 3.12C). Concurrently, there is an increase in active Src levels and talin at the cell leading edges, due to skelemin dissociation. Some of the soluble skelemin can remain bound to $\alpha_{\text{IIb}}\beta_3$ and function as a linker between integrins and the myosin cytoskeleton, thereby transforming it into insoluble skelemin.
CHAPTER 4
EFFECTS OF BIOACTIVE PEPTIDES DERIVED FROM CENTRAL TURN MOTIFS WITHIN CYTOPLASMIC TAILS OF $\alpha_{\text{IIb}}$ AND $\alpha_v$ INTEGRINS

4.1 Introduction:

The binding of integrin to extracellular ligands is not usually constitutive but precisely and strictly regulated by a process known as integrin activation. The regulation of integrin activation is important for physiological and pathological processes such as homeostasis, inflammation, and tumour metastasis. It is clear that the ligand binding capacity of integrin can be switched on through an affinity change mechanism, which is triggered by intracellular signals and transmitted by the cytoplasmic tails of integrin $\alpha$ and $\beta$ subunits (45). The cytoplasmic tails propagate their conformational changes induced by intracellular signals through the transmembrane region, and cause allosteric and long-range conformational rearrangement within the extracellular domain of the receptor dimer (45, 161, 162).

Studies with $\alpha_{\text{IIb}}\beta_3$ integrin found that the membrane-proximal $\alpha$ and $\beta$ cytoplasmic domains forms a ‘clasp’ that maintains integrin in a default low affinity state (36, 39, 158). Cytosolic proteins, such as talin and kindlin cooperatively bind to the membrane-distal region of $\beta_3$ subunits and open the activation-constraining $\alpha$-$\beta$ clasp, triggering integrin activation and ligand binding (163). Cytoplasmic tails of integrin can also regulate integrin-ligand binding by affinity-independent mechanisms,
including integrin clustering, integrin diffusion within the membrane, and linkage of
integrins with the cytoskeleton (162, 164). Integrin lateral diffusion and clustering,
commonly referred as avidity regulation, are secondary events occurring after the
initial integrin affinity change and further increase cell adhesive function (61).

Integrin α-tails are very short and more diverse compared with β-tails. Truncating
almost the entire αIIb-cytoplasmic tail (G991–E1008) constitutively activates αIIbβ3
(37). Cell-permeable peptides derived from full-length αIIb tail (K989–E1008)
inhibited agonist-activated and αIIbβ3-mediated platelet binding to fibrinogen (4).
These data indicate that the α-tail negatively regulates integrin functions. A major
structural feature in α-tails is the central turn motif, which contains a conserved region
in αIIb (RPPL EE) and αV (RPQP EE). NMR structures of αIIb cytoplasmic tails showed
that in this motif, a turn structure formed at the double proline (P998 and P999)
allowing the negatively charged C-terminus of an αIIb tail to fold back and interact
with its N-terminal helix (4). Mutation of both prolines (P998A/P999A) completely
abolished the inhibition of the full-length αIIb tail peptide on integrin activation,
suggesting that the turn motif could be a key inhibitory region. It was supported by
the observations that truncated αIIb tail peptides with the removal of the central and
C-terminal regions activated αIIbβ3 (165, 166), while double mutation (P998A/P999A)
of the αIIb tail expressed in CHO cells rendered αIIbβ3 constitutively active to bind
PAC-1 (anti-αIIbβ3 mAb specifically recognizing active form of αIIbβ3) (46, 167).

To evaluate the contributions of different motifs to the α-tail’s inhibitory effect,
we used cell-permeable peptides that derive from full length or different segments of
The sequences of αIIb and αv cytoplasmic tails and the peptides. (A) The sequences of αIIb and αv cytoplasmic tails. The amino acid sequences used in the experimental peptides are shown in red. (B) Sequences of the αIIb and αv peptides, RPP/AAA mutant and αIIb scrambled peptides.

α-tails (168, 169). The amino-termini of these peptides were coupled to myristic acid, which effectively targets peptides to the plasma membrane and also helps stabilize the secondary structure of flexible peptides (4, 170). We found that myristoylated peptides derived from full-length αV tails inhibited PMA-induced and αvβ3-mediated cell adhesion (168, 169). The turn motif peptide αv (993RVRPPQEEQ) was identified as the minimal inhibitory sequence in αV tail (169). However, the peptide αIIb (995–1003) 995RNRPLEED, which is homologous to αV (993RVRPPQEEQ), was functionally silent for inhibiting αIIbβ3 activation (169). As the removal of several amino acids from the two ends of full-length αIIb tail would not destroy the inhibitory capacity (4), we hypothesize that the turn motif in the αIIb tail is an inhibitory domain, but that extra amino acid(s) outside of αIIb (995–1003) are required for its full inhibitory capacity.
In the present study, myristoylated peptide KRNRPLEED (named as $\alpha_{\text{IIb}}$ peptide, Figure 4.1) was examined in assays of cell adhesion as well as soluble ligand binding in breast cancer cells lines (MDA-MB-435 and MCF-7) and $\alpha_{\text{IIb}}\beta_3$-expressing CHO cells. Including the lysine residue allows introduction of a biotin-tag to the lysine side chain. Cellular proteins that interact with $\alpha_{\text{IIb}}$ peptide were also investigated. The homologous peptide from $\alpha_V$ tails, myr-KRVRPPQEEQ (named as $\alpha_V$ peptide, Figure 4.1) was used in parallel to compare their potency to influent integrin-ligand binding.

4.2 Results

4.2.1 $\alpha_{\text{IIb}}$ and $\alpha_V$ peptides inhibited adhesion of $\alpha_{\text{IIb}}\beta_3$-expressing CHO cells to fibrinogen

To determine whether our $\alpha_{\text{IIb}}$ peptide is inhibitory for $\alpha_{\text{IIb}}\beta_3$-mediated cell adhesion, CHO cells stably expressing $\alpha_{\text{IIb}}\beta_3$ were used (Figure 4.2). Cells were labelled with fluorescent dye Calcein AM and treated with peptides as described in Materials and Methods. Significantly decreased cell adhesion was observed when cells were treated with 50, 100, or 150 $\mu$M $\alpha_{\text{IIb}}$ peptide compared with its scrambled form (Figure 4.2A). At a higher concentration, 200$\mu$M $\alpha_{\text{IIb}}$ peptide showed no difference from the scrambled peptide, possibly because the high concentration of myristoyl group was cytotoxic, leading to dramatic adverse effect on cell adhesion. Figure 4.2B showed that the $\alpha_V$ peptide also had inhibitory effects on CHO cell adhesion at the tested concentrations.
Figure 4.2: Capacity of $\alpha_v$ and $\alpha_{IIb}$ peptides to inhibit $\alpha_{IIb}\beta_3$–overexpressing CHO cell adhesion to fibrinogen. (A) Measurement of cell adhesion when calcein AM-labelled CHO cells were incubated with $\alpha_{IIb}$ or scrambled peptides for 20 minutes at 37°C. (B) Measurement of cell adhesion when $\alpha_{IIb}$, $\alpha_v$ or scrambled peptide was introduced at the indicated concentrations. Treated or untreated cells were added to fibrinogen-coated wells. After adhesion, unattached cells were removed by washing. Adhesion was quantified by fluorescent intensity changes as indicated in Materials and Methods. Adhesion for untreated cells was assigned a value of 100%. The data shown are means and SEM from three different experiments. * P<0.05 vs. scrambled peptide treatment; ** P<0.01 vs. scrambled peptide treatment, by two-way ANOVA. ** P<0.01 compared with scrambled peptide treatment by two-way ANOVA (Bonferroni’s multiple comparisons test).
4.2.2 αIIb and αv peptides inhibited MDA-MB-435 and MCF-7 cell adhesion to vitronectin

We were interested in determining whether the αIIb peptide could inhibit αv integrin-mediated cell adhesion. Two breast cancer cell lines were used, highly metastatic MDA-MB-435 cells and poorly metastatic MCF-7 cells. Peptide effects on the adhesion of both cell types to vitronectin were examined. Vitronectin is a classic αv integrin ligand and it is abundantly expressed in cancer tissues, such as the small vessels surrounding breast cancer cells, and in high-grade glioblastomas (171, 172). αv integrin binding to vitronectin promotes cancer cell survival and migration, and contributes to tumour invasion and metastasis in breast cancer, melanoma and glioma (172-174).

Immunofluorescence images showed that αv integrin binding to vitronectin results in the formation of stress fibers and focal adhesions in both types of breast cancer cells (Figure 4.3). Specifically, many bundles of stress fibers span the core of the MDA-MB-435 cells and distribution of F-actin in some MCF7 cells was condensed and localized at the cell periphery. Focal adhesions visualized by vinculin staining were also highly-developed and exhibited a dot-like distribution pattern. Our observations of the presence of these structures suggest that inhibiting αv integrin-vitronectin binding may potentially target their integrin-mediated migration in the two cancer cell lines.

MDA-MB-435 cells express three αv integrin subfamily members including αvβ3, αvβ5 and αvβ6 (175), and all of them have the capacity to bind vitronectin (176).
Figure 4.4A revealed that both α_{IIb} and α_v peptides inhibited MDA-MB-435 adhesion to vitronectin in a dose-dependent manner. Interestingly the α_{IIb} peptide had a similar inhibitory effect on MDA-MB-435 adhesion as did α_v peptide. Both peptides could inhibit cell adhesion to vitronectin by 50% at a concentration of 8 μM, and totally inhibited cell adhesion at 100 μM. Various function-blocking antibodies were used to determine which of the three present α_v-containing integrins support MDA-MB-435 cell adhesion to vitronectin. Figure 4.4B showed that treatment with anti-α_vβ_3 or α_vβ_5 antibodies, but not anti-α_vβ_6 antibody, could inhibit cell adhesion. Combined treatment with anti-α_vβ_3 and α_vβ_5 antibodies completely prevented cell adhesion. Taken together, these results suggest that α_{IIb} and α_v peptides blocked α_vβ_3 and α_vβ_5-mediated MDA-MB-435 cell adhesion to vitronectin.

To further explore if the peptides are capable of inhibiting both α_vβ_3 and α_vβ_5-dependent cell adhesion, MCF7 cells that do not express α_vβ_3 were used in this assay (175). Both α_{IIb} and α_v appeared to inhibit MCF7 adhesion to vitronectin. Treatment of 25 μM peptide inhibited 50% of the cell adhesion, a lower potency of activity compared with the peptide’s effect on MDA-MB-435 cells (Figure 4.4C). Among the three different function-blocking antibodies, only anti-α_vβ_5 antibody partially inhibited cell adhesion, indicating MCF7 adhesion to vitronectin was primarily mediated by α_vβ_5 integrin (Figure 4.4D). The above data suggest that α_{IIb} and α_v peptides also inhibited α_vβ_5-mediated cell adhesion.
Figure 4.3: Immunofluorescence images showing stress fibers and focal adhesions in MDA-MB-435 and MCF-7 cells grown on vitronectin. Cells were grown on vitronectin-coated chamber slides for overnight, and then fixed, permeabilized and stained with rhodamine phalloidin to detect F-actin-containing stress fibers (red) and anti-vinculin antibody to detect focal adhesions (green).
Figure 4.4: Capacity of αv and αIIb peptides to inhibit MDA-MB-435 and MCF-7 cell adhesion to vitronectin. Calcein AM-labelled cells in suspension were incubated with αv, αIIb and scramble peptides at the indicated concentrations for 20 minutes at 37°C (A, C), or 10 μg/mL of each antibody for 20 minutes at room temperature (B, D). Treated or untreated cells were then added into vitronectin-coated wells. After adhesion, unattached cells were removed by washing. Adhesion was quantified by measuring fluorescence intensity change as indicated in Materials and Methods. Adhesion for untreated wells was assigned a value of 100%. The data shown are means and SEM from three different experiments. Statistical analysis was performed with two-way ANOVA and Bonferroni’s multiple comparisons test in A and C. Statistical analysis with one-way ANOVA and Tukey’s multiple comparisons test in B and D.
4.2.3 $\alpha_{IIb}$ and $\alpha_v$ peptide inhibited the ability of $\alpha_{IIb}\beta_3$-expressing CHO cells to bind soluble fibrinogen

Agonist-stimulated fibrinogen binding to $\alpha_{IIb}\beta_3$ on circulating platelets following vascular injury is critical for platelet aggregation and homeostasis. Wild-type $\alpha_{IIb}\beta_3$ expressed in CHO cells remains in an inactive state as in non-activated platelets. The artificial activator Mn$^{2+}$ was then used to reconstitute integrin activation and peptide effects on Mn$^{2+}$-stimulated $\alpha_{IIb}\beta_3$ binding to soluble fibrinogen were investigated by confocal microscopy. Figure 4.5 showed that CHO cells stimulated with Mn$^{2+}$ displayed a high amount of fibrinogen binding. Clustering of cells upon soluble fibrinogen binding to $\alpha_{IIb}\beta_3$ was visible. In contrast, the presence of $\alpha_{IIb}$ or $\alpha_v$ peptide decreased fibrinogen binding and cell aggregation.

Next, fibrinogen binding levels on CHO cells treated with different concentrations of each peptide were measured by flow cytometry (Figure 4.6). Cells without Mn$^{2+}$ and peptide treatment showed low fluorescence intensity, indicating little background fibrinogen binding. Adding Mn$^{2+}$ promoted substantial fibrinogen binding, reflected by the right shift of fluorescence intensity. Both $\alpha_{IIb}$ and $\alpha_v$ peptide treatment decreased the percentage of cells that bound fibrinogen in a dose-dependent manner. No detectable difference of fluorescence intensity was observed when cells were treated with different concentrations of scrambled peptide (data not shown). These findings provided evidence that $\alpha_{IIb}$ and $\alpha_v$ peptides were able to inhibit Mn$^{2+}$ promoted soluble fibrinogen-$\alpha_{IIb}\beta_3$ binding. However, to address whether findings in CHO cells are relevant to what actually occurs in human platelets, studies
Figure 4.5: Confocal images showing $\alpha_v$ and $\alpha_{\text{IIb}}$ peptide inhibition of Mn$^{2+}$-stimulated soluble fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$-expressing CHO cells. $\alpha_{\text{IIb}}\beta_3$-expressing CHO cells were first treated with 100 $\mu$M peptides, and then stimulated with Mn$^{2+}$ (0.2 mM). Alexa 488-fibrinogen was then added. After washing, cells were fixed and stained for $\beta_3$ integrin. Cells in mounting media were plated on coverslips and visualized by confocal microscopy.
Figure 4.6: Flow cytometric analysis showing $\alpha_v$ and $\alpha_{IIb}$ peptide inhibition of $\text{Mn}^{2+}$-stimulated fibrinogen binding to $\alpha_{IIb}\beta_3$-expressing CHO cells. $\alpha_{IIb}\beta_3$-expressing CHO cells were first treated with peptides at the indicated concentrations, and then stimulated with $\text{Mn}^{2+}$ (0.2 mM). Alexa 488-fibrinogen was then added. After washing, binding of Alexa 488-fibrinogen was measured by flow cytometry. In the figure, the x-axis is fluorescence intensity and the y-axis is forward scattering. A rectangular gate was drawn to indicate dots that were considered to represent positively stained cells. The percentages of positively stained cells were indicated in the rectangular gates.
using thrombin or ADP-activated human platelets need to be carried out.

4.2.4 Peptide mobility on SDS-PAGE gel

The $\alpha_{IIb}$, $\alpha_v$, scrambled peptide and $\alpha_{v\text{mut}}$ (Q998A) were examined on 20% SDS-PAGE gel (Figure 4.7). Staining the polyacrylamide gel with Coomassie blue showed that $\alpha_{IIb}$, $\alpha_v$ and $\alpha_{v\text{mut}}$ peptides migrated with the same apparent molecular weight, whereas the scrambled peptide migrated faster than the other peptides in the gels. Compared with proteins, the intrinsic charge and conformation were found to be important for electrophoretic mobilities of oligopeptides (177). Because $\alpha_{IIb}$ and scrambled peptide had the same molecular weight and intrinsic charge based on their same amino acid composition, their different mobilities on SDS-PAGE gel suggested they may have different conformations. Myristoylated $\alpha_{IIb}$ cytoplasmic tail peptide was reported to form a closed structure conformation highlighted by a turn formed at the double proline in the middle and electronic interactions between the two ends. We expect that the scrambled peptide formed an unstructured conformation as two proline residues were separated and positive charged N-terminus and negative charged C-terminus were also disrupted. A possible reason for the scrambled peptide’s higher electrophoretic mobility is that its disordered form may facilitate a more rapid traverse through the polyacrylamide matrix. From the gel electrophoresis result, it was hypothesized that a closed conformation, lost in the scrambled peptide, may be important for the inhibitory capacity of bioactive $\alpha_{IIb}$ and $\alpha_v$ peptides.
Figure 4.7 The scramble peptide migrates faster than αv and αIIb peptides in SDS gel electrophoresis. 1μg of each peptide was electrophoresed in 20% SDS gels, and the gel was stained with Coomassie blue. Molecular markers are shown left. The molecular weights of the peptides are αIIb 1.46 kDa, αv 1.47kDa, scramble 1.46 kDa.

4.2.5 The turn structure is important for the inhibitory capacity of αIIb peptide

The RPP sequence, conserved in αIIb and αv tails, was predicted to form a β turn structure of the peptides. Previous studies have shown that a double proline mutant of full-length αIIb cytoplasmic peptide was functionally inactive as was the shorter αv peptide (4, 168). We found that mutant peptide with the RPP to AAA (RPP/AAA) substitution attenuated the inhibitory effects of αIIb on cell adhesion. As shown in Figure 4.8A, at a concentration of 8 μM, αIIb peptide treatment inhibited MDA-MB-435 cell adhesion to vitronectin by 58%, significantly greater than the RPP/AAA mutant which showed only 23% inhibition (P<0.01). Similarly, αIIb peptide-induced inhibition on cell adhesion was decreased by RPP/AAA mutant in MCF-7 cells from 53% to 30 % when 25 μM peptides were used, and in αIIbβ3-expressing CHO cells from 49% to 4% when 100 μM peptides were used (Figures 4.8B and 4.8C). These results showed that the RPP/AAA mutant peptide still retained some inhibitory capacity, but with a less potent effect than the αIIb peptide.
Figure 4.8: Decreased inhibitory capacity of the RPP/AAA mutant peptide. Calcein AM-labelled (A) MDA-MB-435, (B) MCF-7, (C) αIIbβ3-expressing CHO cells were treated with αIIb and RPP/AAA mutant peptides at the indicated concentrations, and then added into ligand protein-coated wells. After adhesion, unattached cells were removed by washing. Adhesion was quantified by measuring fluorescence intensity as indicated in Materials and Methods. Adhesion for untreated wells was assigned a value of 100%. The data shown are means and SEM from three different experiments. ** P<0.01 compared with αIIb peptide treatment (two way-ANOVA, Bonferroni’s multiple comparisons test).
4.2.6 Identification of intracellular binding partner

To search for intracellular binding partners associated with the bioactive peptides, we performed immunoprecipitation of total cell lysate from αIIbβ3-expressing CHO cells with the use of biotin tagged αIIb peptide, bio-KRNRPLEED. Biotin-tagged scrambled peptide was used as a negative control. Biotin-tagged peptides were first immobilized onto streptavidin-dynabeads and then incubated with CHO cell lysate. Proteins pulled down via interaction with biotin-tagged peptides were separated on SDS-PAGE gels and visualized by silver staining (Figure 4.9). To search for proteins that specifically interacted with the bio-αIIb peptide, protein bands that were present in higher amount in the bio-αIIb immunocomplex lane than in the bio-scrambled peptide lane were of interest. The 55 kD bands on the SDS-PAGE gel appeared unique in the bio-αIIb immunocomplex (Figure 4.9A). The bands were cut out and identified with mass spectrometry. The putatively identified proteins via MASCOT search are listed in Figure 4.9B. The results indicated that β-tubulin was enriched in the sample pulled down by bio-αIIb peptide. Tubulin-β5 chain and tubulin-4B chain showed higher protein score (protein score is -10×Log(P), where P is the probability that the observed match is a random event). Furthermore, the apparent molecular weight of the bands is 55 kD, which is consistent with the actual molecular weight of tubulin. The overall sequence coverage of tubulin-β5 chain and tubulin-4B chain is presented in Figure 4.9C. 8 peptides matching tubulin-β5 were identified with a protein sequence coverage of 19% (protein sequence coverage is calculated by dividing the number of amino acids observed by the protein amino acid length).
### B

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Figure 4.9: Potential identification of β-tubulin as an αIIb peptide-associated protein with a bio-peptide pull-down assay and mass spectrometry. (A) Peptide pull-down assays using bio-αIIb or bio-scrambled peptide. Bio-αIIb or bio-scrambled peptide was incubated with streptavidin-coated dynabeads. After washing away unbound peptides, the beads were then incubated with αIIbβ3-expressing CHO cell lysate overnight. Proteins interacting with bio-peptides were separated by SDS-PAGE and visualized by silver staining. The bands inside the black rectangle were cut out, digested with trypsin and analyzed by mass spectrometry. (B) MASCOT search results that indicated that β-tubulin was enriched in the sample pulled down by bio-αIIb peptide. Proteins listed in the column have protein scores higher than the threshold, which can be considered as identified (p<0.05). (C) Sequence of tubulin beta-5 and beta-4B chain. Recovered peptides matching the sequences of tubulin beta-5 (top sequence) and tubulin beta-4B chain (bottom sequence) are depicted in dark, whereas regions not identified by recovered peptides are shown in gray.
4.2.7 αv and αIIb peptides did not change the levels of p-ERK and pY397-FAK in MDA-MB-435 cells

As our mutations of integrin cytoplasmic tails did not affect downstream signalling through FAK in CHO cells (Figure 3.7A), we then examined the role of the central turn motif of α-tails by studying the effects of peptides on integrin outside-in signalling through FAK and ERK in MDA-MB-435 breast cancer cells. Serum-starved cells were trypsinized and suspended in Tyrodes buffer. Cells in suspension were treated with or without peptides for 20 minutes and then plated on vitronectin. After one hour of adhesion, cells were solubilised and the levels of p-ERK and pY397-FAK were studied by Western blot. Figure 4.10 shows that cells contained constitutively activated ERK and FAK by measuring the levels of p-ERK and pY397-FAK in non-adherent suspension cells (the first lanes of the blots). Cell adhesion on vitronectin increased p-ERK levels only slightly compared with cells in suspension. Treatment with scr, αIIb or αv peptide did not cause any observable changes of p-ERK levels in the adherent cells (Figure 4.10A). Similar results were obtained for pY397-FAK. As shown in Figure 4.10B, pY397-FAK levels showed little difference when comparing adherent to non-adherent cells, and pY397-FAK remained at the same level in adherent cells treated with αIIb, αv or scrambled peptide.
Figure 4.10  $\alpha_v$ and $\alpha_{\text{IIb}}$ peptides did not change the levels of p-ERK and pY397-FAK in MDA-MB-435 cells. Serum-starved MDA-MB-435 cells were exposed to $\alpha_{\text{IIb}}$, $\alpha_v$ and scramble peptide (100 $\mu$M each) for 20 minutes, and allowed to adhere on vitronectin for 2 hours. Total cell extracts were subjected to SDS-PAGE. Blots were probed using antibodies against pERK (P42/P44), ERK, Y397-FAK, FAK and $\beta$-actin.
4.2.8 Post-treatment with αIIb peptide did not reverse ligand-integrin engagement

Our data showed that αIIb and αv peptides were potent integrin antagonists when they were pre-treated. Thus, a new question is raised whether the peptides still possess inhibitory capacity after the integrin is activated and ligand engagement has occurred. The functional consequences of peptide treatment before and after integrin activation were compared using platelet aggregation and cell adhesion experiments. Platelet aggregation assays were carried out in an aggregometer, which measures light transmission changes of sample solution caused by platelet aggregation. As shown in Figure 4.11A, adding agonists ADP/EPI induced platelet aggregation which lead to less light absorption and increased transmission as recorded in the aggregometer. Pre-incubation with 50 μM αIIb full-length CT (cytoplasmic tail) peptide before the addition of agonists effectively blocked platelet aggregation, indicated as the decreased light transmission (Figure 4.11A). However, post-treatment with αIIb CT peptide had no inhibitory effect on platelet aggregation (Figure 4.11B).

We also found that the central turn peptides, αIIb and αv, could not detach αIIbβ3-expressing CHO cells adhered on fibrinogen (Figure 4.11C). Cells were first allowed to adhere to integrin ligand for one hour, and then treated with buffer or 100 μM αIIb, αv or scramble peptide. After one more hour of adhesion, unattached cells were removed by washing. Cell adhesions in the presence of buffer were displayed as 100%. No statistically significant differences were observed between the buffer and peptide-treated groups ((p<0.05), suggesting that neither αIIb nor αv peptide was able to detach αIIbβ3-expressing CHO cells from fibrinogen. Similar results were obtained
for adherent MDA-MB-435 and MCF-7 cells (Figure 11 D and E).

Figure 4.10 Treatment of the inhibitory αIIb or αv peptides did not reverse ligand-integrin engagement. Effect of pre-treatment (A) and post-treatment (B) of αIIb CT peptide on platelet aggregation stimulated by ADP/EPI. (A) PRP was stimulated with ADP/EPI to induce platelet aggregation (indicated as ADP/EPI). 50μM αIIb CT peptide-treated PRP were stimulated with ADP/EPI to generate an inhibition curve (indicated as αIIb CT). PP/AA mutant peptide was used as a control. (B) PRP was treated with 50μM αIIb CT peptide or control peptide five minutes after the agonists were added. No inhibition of platelet aggregation was observed. (C, D and E) Neither αIIb nor αv peptide was able to detach αIIbβ3-expressing CHO cell
adhesion on fibrinogen (C), and MDA-MB-435 (D) and MCF-7 (E) cell adhesion on vitronectin. Cells were harvested, labelled by Calcein AM, and allowed to adhesion on integrin ligand in Tyrodes buffer for one hour. 100μM αIIb, αv, or scramble peptides were then added. After one more hour adhesion, unattached cells were removed by washing. Adhesion was quantified by measuring fluorescence intensity before and after wash as indicated in Materials and Methods. The buffer bars represent untreated groups, which were assigned a value of 100%. The data shown are means and SEM from three different experiments. Student’s t-test was performed and no statistically significant difference was found between the buffer and peptide-treated groups (p<0.05).

4.3 Discussion

The full-length cytoplasmic tail peptides from αIIb (989-1008) and αv (987–1006) suppressed the ligand binding function of αIIbβ3 and αvβ3 integrins, respectively (169). We previously found that peptide RVRPPQEEQ (993-1001), corresponding to the central motif of αv tail, inhibited αvβ3 and αIIbβ3 ligand binding, and it appeared to be the shortest sequence among truncated cytoplasmic tail peptides that still retained the inhibitory capacity. However, its homologous peptide RNRPPLEED (995-1003) derived from αIIb tails was functionally silent for inhibiting αIIbβ3-mediated fibrinogen binding to platelets (169). In the present study, αIIb peptide KRNRPPLEED (994-1003) that include the adjacent lysine residue was examined. We found that it blocked αIIbβ3-expressing CHO cells binding to immobilized or soluble fibrinogen (Figures 4.2, 4.5 and 4.6). This work suggests the αIIb central turn motif is the inhibitory domain of the αIIb cytoplasmic tail and that the residue lysine (K994) is required for full inhibitory capacity.

More importantly, we found that the homologous peptides αIIb (994-1003) and αv (992-1001) suppressed both αIIbβ3 and αvβ3 functions, which may lead to a new
therapeutic strategy for an anti-cancer and anti-platelet dual-role drug. Our work showed that peptides αIIb (994-1003) and αV (992-1001) were equally effective at inhibiting αIIbβ3 and αVβ3 mediated-cell adhesion to the immobilized and soluble ligands (Figures 4.2 and 4.4), although peptides derived from the full-length αIIb and αV tails can only block their parent integrin’s functions. A previous study showed that αIIb (989-1008) tail peptide only blocked αIIbβ3 activation, and αV (987–1006) tail peptide only blocked αVβ3 activation (169). The functional specificity of the full-length tails may attribute to their membrane proximal regions. Cytoplasmic tails of αIIb and β3 subunits interact to form a complex through their membrane-proximal regions, which maintains integrin αIIb and β3 in an inactive state (22, 23). A possible mechanism for the inhibitory effect of the full-length tail peptides is due to their competition with the native tails of their parent integrins in binding to β tails (4). Our recent work using surface plasmon resonance (SPR) spectroscopy found that the αV and β3 tails also interacted with each other (169). However, the αV turn motif peptide (993–1001) had low affinity for β3 tails and was ineffective at perturbing of αV-β3 tail interaction (169). Moreover, the highly conserved KXGFFKR motif in the membrane proximal region acts as a recognition site for multiple intracellular proteins. Studies using protein chips have identified 68 direct binding proteins to this conserved motif derived from different α integrin isoforms (47). However, most of these binding proteins are specific for different integrin family members (47), suggesting that the protein binding sites in membrane proximal region are different among α integrin isoforms.
The conserved amino acid composition and similar activity of α\textsubscript{IIb} and α\textsubscript{V} turn peptides indicate that the same functional mechanism occurs with both peptides. The cytoplasmic tail of the integrin α\textsubscript{IIb}-subunit has often served as a typical prototype for structure-function analyses (45). Here we used the wild-type, mutant and scrambled α\textsubscript{IIb} turn motif peptides to investigate the possible mechanism shared by the α\textsubscript{IIb} and α\textsubscript{V} turn motifs.

RPP residues are important for maintaining the backbone structure of the turn. The NMR structure of the α\textsubscript{IIb} tail (PDB# 1DPK) showed that the turn motif allowed the acidic C-terminus to fold back and interact with the positively charged N-terminal, resulting in a “closed” conformation. However, mutation of PP (α\textsubscript{IIb} 998,999) to AA (PDB# 1DPQ) disrupted the turn and opened the closed conformation (4). This double proline mutant peptide was inactive in α\textsubscript{IIb} inhibition (4). Moreover, D-isomeric replacement of the α\textsubscript{V} (993-1001) at RPPQ abolished its capacity to block integrin activation (169). The L- to D-amino acid conversion in the α\textsubscript{V} tail may reverse the direction of the turn fold and the spatial orientation of the side chains. Similarly, we found that, compared to its original α\textsubscript{IIb} peptide (994-1003), the mutant peptide RPP/AAA had significantly decreased capacity for inhibiting the adhesion of MDA-MB-435, MCF-7 and α\textsubscript{IIb}β\textsubscript{3}-expressing CHO cells (Figure 4.7). The α\textsubscript{IIb} peptide (994-1003) KRNRPPLEED retains the elements that facilitate formation of a turn structure: positive charged lysine and arginine residues at the N-terminus, negative charged glutamic acid and aspartic acid residues at the C-terminus, and double proline residues in the middle. In addition, K994 is possibly important for the activity of the
αIIb peptide because the turn structure is stabilized by K994 binding with the negatively charged C-terminus of the peptide.

The NMR structure of αIIb (989–1008) and molecular models of αv (987–1018) suggest that the central turn motif is highly exposed and accessible to intracellular binding partners (168, 169). Thus, it is possible that the inhibitory activity of the turn peptides is due to their competition with the native turn in binding to these partners. In the present study, we used bio-peptide pull-down assay followed by MS/MS peptide sequencing to identify β-tubulin as a potential binding partner with the bio-αIIb peptide. Although a large number of proteins were pulled down, the protein band identified to be β-tubulins were enriched in bio-αIIb pull-down samples, indicating its higher binding affinity for αIIb peptide than scrambled peptide. β-tubulin has been previously shown to have high binding affinity for integrin KVGFFKR regulatory motif, the N-terminus of αIIb cytoplasmic tails (47). The current study potentially identified the binding of β-tubulin to the central turn motif KRNRPPLLEED, where the first two amino acids (KR) overlap with KVGFFKR. Two β-tubulin-binding motifs within αIIb tails suggest a potential role of β-tubulin in integrin function through binding with αIIb tails. β-tubulin is a microtubule component. Microtubules function in intracellular organelle transport, mechanical support of cells and cell mitosis. Interestingly, microtubules have also been shown to affect integrin β2 avidity regulation (178), cell adhesion (179, 180) and cell spreading (64). The data presented here were consistent with the previous report that pharmacological disruption of microtubules inhibited early adhesive interactions between circulating tumour cells and host organ
microvasculature in vivo (179). Our findings should improve our understanding of the importance of microtubule-integrin linkage in integrin activation regulation and integrin-mediated adhesion.

Interestingly, we also found that \( \alpha_{IIb} \) and \( \alpha_V \) peptides inhibited Mn\(^{2+}\)-stimulated soluble fibrinogen binding to \( \alpha_{IIb}\beta_3 \)-expressing CHO cells. First, confocal images showed that there was less labelled fibrinogen binding in cells treated with \( \alpha_{IIb} \) or \( \alpha_V \) peptide, and cell aggregation mediated by multivalent fibrinogen linking adjacent cells (Figure 4.5). Second, flow cytometry confirmed that \( \alpha_{IIb} \) inhibits soluble fibrinogen binding in a dose dependent manner using scrambled peptide as a control (Figure 4.6). Mn\(^{2+}\) replaces an inhibitory Ca\(^{2+}\) at the adjacent metal ion-binding site in integrin ectodomain and induces an extended and high-affinity integrin conformation (181, 182). Recent studies using living cells demonstrated that the conformational changes induced in the ectodomain by Mn\(^{2+}\) can be transmitted toward the transmembrane and cytoplasmic domains where integrin oligomers or clusters are formed, even in the absence of extracellular ligands (183-185). Integrin clustering increases the local density of integrins and recruits adaptors or cytoskeletal proteins to form adhesions which link cytoskeleton to extracellular matrix (3). Given that myristoylation allows delivery of the experimental peptides into cells and that nonmyristoylated peptides causes little inhibition (4, 169), our work indicates that the inhibitory effect of the \( \alpha_{IIb} \) peptide likely occurs by disturbing Mn\(^{2+}\)-induced intracellular integrin clustering. Previous work found that integrin clustering did not spontaneously occur when integrin affinity increased, but required a driving force
from integrin binding partner, such as talin (185). Our data suggest that αIIb peptide may inhibit the interactions between αIIb tail and its binding proteins. However, whether β-tubulin is actually an integrin binding protein and target of αIIb turn peptide remains further investigation.

Our observations showed that treatment of the agonist-stimulated platelets and adhered cells with the αIIb and αV peptides did not reverse ligand-integrin engagement. The initiating step in integrin activation is a conformational change in the integrin cytoplasmic tails and the transmembrane domains. The conformational changes then propagate from the transmembrane domains to the ligand binding headpiece, which increases integrin affinity for ligand. The data here indicates that the CT peptides can block the initiating event. However, once it has occurred it cannot be reversed as the numerous interactions that occur following ligand binding to stabilize the ligand-bound state.

In summary, the current work suggests that the central turn motif in αIIb and αV tails is not only a structural support, but also an important protein anchoring site. Some cytosolic proteins, such as β-tubulin, may recognize and bind to the central turn, participating in control of integrin activation. These binding proteins function as activators which change α tail’s conformation (for example, opening the “closed” conformation), or promote integrin clustering. αIIb and αV turn peptide may inhibit integrin activation as a result of sequestration of the cytosolic factors.
Bidirectional signalling across integrin receptors includes both ‘inside-out’ and ‘outside-in’ signalling pathways. Intracellular signals trigger direct interactions of β cytoplasmic tails with talin and kindlin, leading to tail separation and conformational changes in the extracellular domain of integrin (integrin activation or inside-out signalling). Following ligand binding, integrins transduce signals into cells (outside-in signalling) by recruiting proteins to their cytoplasmic tails, which results in actin reorganization and modulation of signalling pathways. The cytoplasmic tails of integrins are short, but they serve as receivers and transmitters of the bidirectional signalling and are involved in assembly and disassembly of many binding partners. Despite years of extensive studies on specific amino acids, motifs within integrin cytoplasmic tails and their binding partners involved in integrin bidirectional signalling, a number of key questions still remain (186). What is the role of the distal region of α and β cytoplasmic tails in integrin activation? What are the precise molecular linkages between integrin cytoplasmic tails and the cytoskeleton? How do these interactions regulate cell adhesion, spreading and migration? Two main experimental approaches have been used to address these questions, specifically the expression of αIIBβ3 in heterologous cells, and the introduction of membrane permeable peptides derived from the segments of cytoplasmic tails.

We began with an investigation of the role of skelemin-integrin binding in
integrin activation and cell spreading using protein mutagenesis (Chapter 3). Wild-type or mutant αIIbβ3 receptors defective in skelemin binding were stably expressed in CHO cells and their inside-out and outside-in signalling were examined.

A previous NMR spectrometry study showed that skelemin can bind with both αIIb and β3 tails and had a weak capacity to separate the complex of αIIbβ3 cytoplasmic tails, which raises a question whether skelemin can activate integrins by unclasp the interface of αIIb and β3 tails. The current work showed that overexpression of the integrin-binding domain of skelemin (skeC2) neither activated wild-type αIIbβ3, nor changed the affinity state of mutant integrins in a PAC-1 binding assay. It suggests that skelemin cannot unclasp the membrane-proximal interface of αIIbβ3 cytoplasmic tails in CHO cells, even though skelemin binds both of the subunits, as does the head domain of talin. This result is consistent with previous immunoprecipitation results showing that associations of skelemin and integrin αIIbβ3 only occur following integrin ligation in platelets and CHO cells. It fortifies the conclusion that skelemin is not an upstream activator of inside-out signalling, but is more likely to be a downstream effector following integrin activation or inside-out signalling.

Integrin outside-in signalling can be evaluated by cell spreading. A previous view regarded skelemin as an essential protein linking integrins to the cytoskeleton was based on the observation that microinjection or overexpression of skeC2 fragments into cultured cells abolished cell spreading. The skeC2 fragments were thought to do so by competing with endogenous skelemin binding to integrins and breaking the integrin-cytoskeleton linkage. Similar results were obtained here with the use of CHO
cells and Hek293 cells, which showed inhibited αIIbβ3-mediated cell spreading on fibrinogen after co-transfection with skeC2 and αIIbβ3 integrin. However, that view is challenged by the observation that mutant αIIbβ3-transfected cells have unchanged or even increased cell adhesion and spreading capacity at the early stages of cell spreading on immobilized fibrinogen. These cells formed membrane protrusions, focal adhesions and stress fibers, suggesting unimpaired integrin outside-in signalling leading to focal adhesion assembly and actin polymerization. These data lead us to conclude that engagement of skelemin to the cytoplasmic tail of αIIbβ3 is not essential for cell spreading.

Some of the integrin mutant cells, namely H722A and K716A had a larger cell spreading area. This is in agreement with their elevated levels of pY416-Src which are required for integrin-mediated cell spreading. The immunofluorescence study further investigated K716A mutation-expressing cells, as the K716 residue appeared to be the most important for skelemin binding in in vitro studies. It showed that wild-type integrins were colocalized with both skelemin and talin in the cell periphery at the early stage of cell spreading, whereas K716A cells showed strong colocalization of talin with mutated αIIbβ3 in the cell protrusions even with a loss in skelemin binding. These results suggest that binding of skelemin and talin to the membrane proximal region of β3 tails may be mutually exclusive, and that disrupting skelemin binding could facilitate talin recruitment that promotes actin polymerization and formation of membrane protrusions.

We hypothesize that the skelemin-integrin interactions function to coordinate the
binding of different cytoskeletal proteins to the membrane proximal region of integrin tails, such as talin. As a member of the family of myosin-associated proteins, skelemin was thought to exert a contractile force by linking integrin to myosin. The enhanced cell spreading in some mutant cells support this view. Thus, modulating skelemin binding to integrin tails is one mechanism a cell can use to fine tune the highly organized process of cell spreading.

Meanwhile, this work also shows that the stoichiometry of skelemin and integrin is important for integrin-mediated function. We found that GFP-skeC2 inhibited the αIIbβ3-mediated cell spreading only when the ratio of GFP-skeC2 to αIIbβ3 was high. However, if the ratio of skeC2 to integrin is much higher in plasmid-transfected CHO cells than it is in wild type cells, such as platelets, this conclusion about skelemin’s function could be over-evaluated or even wrong, as the over-expressed skeC2 may saturate the skelemin binding sites of integrin and affect the association of integrin with other integrin binding partners that compete with skelemin. Therefore, we think that studies utilizing the transfection of wild-type and mutant integrin may offer an alternative, and perhaps better, way to evaluate the function of skelemin than simple overexpression of skelemin.

Platelets are anucleate and therefore not amenable to direct genetic manipulations. CHO cell lines expressing wild-type and mutant αIIbβ3 have been successfully used to study the role of individual amino acids and specific interactions in integrin bidirectional signalling. Since the experiments reported here are done with CHO cells, the question arises whether the conclusions reached will apply to other cell types and
platelets. Transfection of primary megakaryocytes, the precursor of platelets, and generation of mice bearing mutant α<sub>IIb</sub>β<sub>3</sub> integrin may be considered in the future for a better understanding of the significance of skelemin in α<sub>IIb</sub>β<sub>3</sub> biology.

Chapter 4 examines how the central motif of the α<sub>IIb</sub> tail regulates integrin activation and integrin-mediated cell adhesion. Previously, we located the minimal α<sub>V</sub> cytoplasmic tail sequence that suppressed integrin activation at the central turn region. The central motifs of the integrin α<sub>IIb</sub> and α<sub>V</sub> cytoplasmic tails are highly conserved. The present study showed that a cell-permeable peptide corresponding to the central motif of the α<sub>IIb</sub> tail, myr-KRNRPPLEED (α<sub>IIb</sub> peptide) inhibited α<sub>IIb</sub>β<sub>3</sub>-mediated cell adhesion to fibrinogen. It also inhibited α<sub>V</sub>β<sub>3</sub> and α<sub>V</sub>β<sub>5</sub>-mediated breast cancer cell (MDA-MB-435 and MCF7) adhesion to vitronectin. The homologous peptide derived from α<sub>V</sub> tails, myr-KRVRPPQEEQ (α<sub>V</sub> peptide) inhibited these cell adhesive functions with a similar capacity, suggesting that the two inhibitory peptides share a common functional mode. Structural studies show that the RPP residues are important for maintaining the turn structure of the α<sub>IIb</sub> tail peptide (4). In this project, replacement of RPP with AAA significantly attenuated the inhibitory activity of the α<sub>IIb</sub> peptide, suggesting that the turn structure formed by PP is important for its inhibitory capacity. The turn structure could be an anchoring point for some cytosolic factors to regulate integrin activation.

In addition, α<sub>IIb</sub> peptide blocks soluble fibrinogen binding of α<sub>IIb</sub>β<sub>3</sub> integrins if they are activated from outside the cell by Mn<sup>2+</sup>. This result suggests that the peptide can interfere with the allosteric conformational changes in integrin cytoplasmic
domain induced by Mn$^{2+}$. One possible mechanism is that the α$_{IIb}$ peptide may disturb Mn$^{2+}$-induced integrin clustering in the presence of fibrinogen.

The peptide pull-down assay identified β-tubulin as a potential novel binding partner with the central turn motif KRNRPLEED within α$_{IIb}$ tails. However, this experiment was done only once and needs to be reproduced. In addition, the interaction between β-tubulin and α$_{IIb}$ peptide needs to be confirmed with other biochemical studies, such as SPR or co-immunoprecipitation. Moreover, whether any potential peptide binding with β-tubulin contributes to its inhibitory capacity was not addressed. The role of β-tubulin in integrin activation and/or integrin clustering requires extensive further studies.

Integrin α$_{IIb}$β$_3$ has a critical role in thrombosis and haemostasis. Active α$_{IIb}$β$_3$ expressed on platelets binding to fibrinogen in plasma leads to platelet aggregation, an important process to stop bleeding at the site of vascular injury. In contrast, abnormal integrin activation may result in life-threatening platelet aggregation and thrombosis formation. α$_{IIb}$β$_3$ antagonists are widely used for the treatment of acute coronary syndromes. However, they have only shown significant promise with percutaneous coronary interventions. The reason for their limited use in the clinic may be due to their activating effects on platelet aggregation and thrombosis at low concentrations (135). Future antagonists will likely be designed for use as anti-platelet agents with the rational of maintaining α$_{IIb}$β$_3$ in its quiescent, noncompetent state. One possible approach is targeting to the intracellular domain of integrin α$_{IIb}$β$_3$ (7). Our peptides corresponding to the central turn motifs of cytoplasmic tails of α$_{IIb}$ and α$_V$ are shown
to inhibit $\alpha_{\text{IIb}}\beta_3$-mediated fibrinogen binding, and these inhibitory peptides may be a starting point for development of anti-thrombotic drugs.

Thrombosis is also a common complication of patients with cancer (187). Cancer patients have an increase in the risk for developing thrombosis events, both in arteries and in veins (188, 189). Multiple and complex mechanisms contribute to cancer-related thrombosis, including tissue factor released by tumour cells, production of MP and inflammatory cytokines by tumour and/or host cells, and direct adhesion of tumour cells to platelets, leukocytes, and endothelial cells (187). These clot-promoting factors in turn contribute to tumour progression and metastasis. Platelets appear to be important for tumour progression as platelet-tumour cell interaction can protecting tumour cells from clearance by immune cells and facilitate their arrest in the vasculature, inducing subsequent tissue invasion (16, 190-192). Fibrinogen functions as a bridge between integrins $\alpha_{\text{IIb}}\beta_3$ on platelets and $\alpha_{\text{V}}\beta_3$ on tumour cells (191). In a mouse model, $\alpha_{\text{IIb}}\beta_3$ antagonists showed potential for blocking tumour metastasis (193). These results indicate that the combined blockade of $\alpha_{\text{V}}\beta_3$ and $\alpha_{\text{IIb}}\beta_3$ may be effective in inhibiting tumour progression. Administration of abciximab, which binds both $\alpha_{\text{V}}\beta_3$ and $\alpha_{\text{IIb}}\beta_3$ with equivalent affinity, showed increased anti-angiogenic and anti-tumour effects compared with blocking tumour integrin $\alpha_{\text{V}}\beta_3$ alone (194). A recent study showed that anti-angiogenic drugs could increase invasive and metastatic properties of breast cancer cells via hypoxia-response program (195, 196). Therefore, combination of anti-angiogenic drugs with others targeting tumour metastasis may improve clinical outcome. $\alpha_{\text{V}}$ integrins promote
tumour initiation, progression and metastasis by regulating tumour cell proliferation, migration and invasion, angiogenesis and ECM remodelling \cite{129}. These studies suggest that the homologous peptides $\alpha_{\text{IIb}}$ (994-1003) and $\alpha_{\text{V}}$ (992-1001) with similar inhibitory activity for $\alpha_{\text{IIb}}\beta_3$ and $\alpha_{\text{V}}$ functions may lead to a new therapeutic strategy for an anti-cancer and anti-platelet dual-role drug. This holds clinical promise for cancer patients at high risk of developing thrombosis.
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