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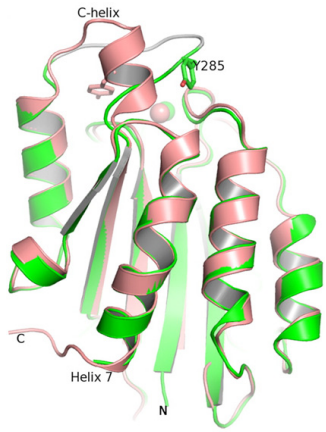
THE STRUCTURE AND FUNCTION OF αI DOMAINS IN COLLAGEN RECEPTOR AND LEUKOCYTE INTEGRINS

by

Matti Lahti



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Dedicated to the memory of
my dear brother Juho
(1976 - 2009)

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ABSTRACT

Integrins are heterodimeric, signaling transmembrane adhesion receptors that connect the intracellular actin microfilaments to the extracellular matrix composed of collagens and other matrix molecules. Bidirectional signaling is mediated via drastic conformational changes in integrins. These changes also occur in the integrin αI domains, which are responsible for ligand binding by collagen receptor and leukocyte specific integrins. Like intact integrins, soluble αI domains exist in the closed, low affinity form and in the open, high affinity form, and so it is possible to use isolated αI domains to study the factors and mechanisms involved in integrin activation/deactivation. Integrins are found in all mammalian tissues and cells, where they play crucial roles in growth, migration, defense mechanisms and apoptosis. Integrins are involved in many human diseases, such as inflammatory, cardiovascular and metastatic diseases, and so plenty of effort has been invested into developing integrin specific drugs.

Humans have 24 different integrins, four of which are collagen receptor ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$) and five leukocyte specific integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_E\beta_7$). These two integrin groups are quite unselective having both primary and secondary ligands. This work presents the first systematic studies performed on these integrin groups to find out how integrin activation affects ligand binding and selectivity. These kinds of studies are important not only for understanding the partially overlapping functions of integrins, but also for drug development. In general, our results indicated that selectivity in ligand recognition is greatly reduced upon integrin activation. Interestingly, in some cases the ligand binding properties of integrins have been shown to be cell type specific. The reason for this is not known, but our observations suggest that cell types with a higher integrin activation state have lower ligand selectivity, and vice versa. Furthermore, we solved the three-dimensional structure for the activated form of the collagen receptor α_1I domain. This structure revealed a novel intermediate conformation not previously seen with any other integrin αI domain. This is the first 3D structure for an activated collagen receptor αI domain without ligand. Based on the differences between the open and closed conformation of the αI domain we set structural criteria for a search for effective collagen receptor drugs. By docking a large number of molecules into the closed conformation of the α_2I domain we discovered two polyketides, which best fulfilled the set structural criteria, and by cell adhesion studies we showed them to be specific inhibitors of the collagen receptor integrins.

Matti Lahti (2012) Kollageenireseptori- ja leukosyytti-integriinien αI-domeenien rakenne ja toiminta. Biokemian ja elintarvikekemian laitos ja Turun biolääketieteellinen tohtoriohjelma, *Turun yliopiston julkaisuja,* **sarja A1, 453.**

TIIVISTELMÄ

Integriinit ovat kahdesta erilaisesta alayksiköstä koostuvia, solukalvon läpäiseviä, signaalivälitykseen osallistuvia reseptoriproteiineja, jotka liittävät solunsisäisen aktiinimikrofilamenttitukirangan solun ulkopuoliseen proteiiniverkostoon. Kaksisuuntainen signalointi välittyy integriineissä tapahtuvien suurten rakennemuutosten kautta. Kollageenia sitovissa ja leukosyyteille ominaisissa integriineissä on αI-domeeni, joka vastaa näihin integriineihin kiinnittyvien proteiinien (ligandien) sitomisesta. Solukalvolla olevien integriinien tavoin eristetyt, liukoiset αI-domeenit esiintyvät sekä suljetussa, pienen aktiivisuuden omaavassa muodossa että avoimessa, suuren aktiivisuuden muodossa. Näin ollen liukoisilla αI-domeeneilla voidaan tutkia integriinien aktivaatioon liittyviä tapahtumia. Integriinejä on nisäkkäiden kaikissa soluissa ja ne ovat keskeisiä solujen kasvussa, liikenteessä, puolustusmekanismeissa ja ohjelmoidussa solukuolemassa. Integriineillä on merkitystä monissa taudeissa, kuten tulehdus- ja verisuonitaudeissa sekä syövissä, ja siksi viime vuosina on runsaasti panostettu tiettyjen integriinien toimintaa estävien lääkeaineiden kehitystyöhön.

Ihmisellä on 24 erilaista integriiniä. Väitöskirjassa tutkittiin ihmisen kollageenireseptori-integriinejä, joita on neljä ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$), ja leukosyytti-integriinejä, joita on viisi ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_E\beta_7$). Nämä integriinit sitovat melko epäselektiivisesti erilaisia molekyylejä. Ensimmäistä kertaa näillä integriineillä tutkittiin systemaattisesti aktivoitumisen vaikutuksia ligandien sitomiseen ja ligandiselektiivisyyteen. Väitöskirjatyön tulokset ovat tärkeitä paitsi näiden integriiniperheiden osittain päällekkäisten toimintojen ymmärtämiselle, mutta myös integriineihin kohdistuvalle lääkekehitykselle. Tulokset osoittavat, että integriinien aktivoituessa niiden kyky tunnistaa ligandeja heikkenee. Joidenkin integriinien sitomisominaisuuksien on havaittu riippuvan solutyypistä. Syytä tähän ei tiedetä, mutta tulostemme perusteella suuren integriiniaktiivisuuden omaavat solut saattavat pystyä sitomaan useammanlaisia proteiineja kuin pienen aktiivisuuden solut. Määritimme kollageeni-integriinin aktivoidun α_1I-domeenin kolmiulotteisen rakenteen, joka osoittautui aiemmin tuntemattomaksi, αI-domeenin suljetun ja avoimen rakenteen välimuodoksi. Tämä on ensimmäinen kollageenireseptori-integriinin aktivoidun αI-domeenin rakenne ilman ligandia. Lääkekehitysprojektiin liittyen löysimme αI-domeenin suljetun ja avoimen rakenteen välisiin eroihin perustuen kaksi polyketidien luokkaan kuuluvaa yhdistettä, jotka sitoutuvat tehokkaasti kollageeni-integriinien suljettuun muotoon estäen niiden toiminnan.

TABLE OF CONTENTS

ABBREVIATIONS

ADMIDAS	Adjacent to MIDAS
3D	Three-Dimensional
cDNA	Complementary deoxyribonucleic acid (DNA)
CHO	Chinese hamster ovary
CR3 and CR4	Complement receptors 3 and 4
ECM	Extracellular matrix
EM	Electron microscopy
GFOGER	Recognition sequence in collagen where O is hydroxyproline
GST	Glutathione-S-transferase
iC3b	Human inactivated complement fragment 3
ICAM-1	Intercellular cell adhesion molecule 1
K_d	Dissociation constant of protein-ligand complex (M)
kDa	kilodalton
LIMBS	Ligand-associated metal binding site
MIDAS	Metal ion-dependent adhesion site
NMR	Nuclear magnetic resonance
RGD	Recognition sequence Arg-Gly-Asp of some ligands
RNA	Ribonucleic acid
TMD	Transmembrane domain
VWA	von Willebrand A domain
$\alpha_{1\text{-}11}$, $\alpha_{1Ib,V,L,M,X,D,E}$	Different integrin α subunits
$\beta_{1\text{-}8}$	Different integrin β subunits
αI-less integrins	Integrins lacking αI domain
αI, β-propeller, thigh, and calf1-2	The extracellular domains of integrin α subunits
PSI, hybrid, βI (= βA), EGF1-4, and βTD	The extracellular domains of integrin β subunit

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles and manuscript, which are referred to by their Roman numerals in the text.

I Lahti M., Bligt E., Niskanen H., Brandt A., Parkash V., Jokinen J., Patrikainen P., Käpylä J., Heino J. & Salminen T. (2011) Structure of collagen receptor integrin α_1I domain carrying the activating mutation E317A. *J. Biol. Chem.* **286**, 43343-43351.

II Tulla M., Lahti M., Puranen S., Brandt A.-M., Käpylä J., Domogatskaya A., Salminen T., Tryggvason K., Johnson M.S. & Heino J. (2008) Effects of conformational activation of integrin α_1I and α_2I domains on selectivity recognition of laminin and collagen subtypes. *Exp. Cell Res.,* **314**, 1734-1743.

III Käpylä J., Pentikäinen O.T., Nyrönen T., Nissinen L., Lassander S., Jokinen J., Lahti M., Marjamäki A., Johnson M.S. & Heino J. (2007) Small molecule designed to target metal binding site in the α_2I domain inhibits integrin function. *J. Med. Chem.,* **50***,* 2742-2746.

IV Lahti M., Heino J. & Käpylä J. (2012) Leukocyte integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ as collagen receptors – Receptor activation and recognition of GFOGER motif. *Manuscript submitted for publication.*

1. REVIEW OF THE LITERATURE ON INTEGRINS

1.1. INTRODUCTION

1.1.1. Integrins

Integrins are bidirectionally signaling transmembrane receptors composed of two different subunits, α and β (Hynes, 1992). They integrate the intracellular cytoskeleton, the contractile actin microfilament system and signaling pathways to the network of extracellular matrix (ECM) proteins (Brakebusch & Fässler, 2003; Humphries et al., 2004; Kinashi, 2005). In vertebrates specific integrins are also involved in cell-cell adhesion interacting with other transmembrane proteins of the neighbouring cells. In order to function properly it is absolutely essential that integrins become active and thus capable of binding their ECM-ligands only under specific conditions (Hynes, 2002; Kim et al., 2011). Bidirectional signaling and the regulation of integrin activity are mediated via large conformational changes (Takagi et al., 2002; Hynes, 2004; Mould & Humphries, 2004a; Arnaout et al., 2005; Gahmberg et al., 2009; Moser et al., 2009; Shattil et al., 2010; Springer & Dustin, 2012) (Figure 1).

Integrins are found in all multicellular animals, but not in prokaryotes, plants, or fungi. The number of different integrin α and β subunits varies in different species. Mammals have 24 different heterodimeric integrins composed of eight different β subunits and eighteen different α subunits. Although some subunits appear only in a single heterodimer, 12 integrins contain a β_1 subunit and five have α_V, for example (Figure 2). The occurrence of integrins in all multicellular animals suggests that integrins are essential for the development of multicellular organs and tissues (Hynes, 2002; Kim et al., 2011).

Integrins are divided into five groups: collagen-binding integrins (section 1.1.2.), laminin binding integrins (Nishiuchi et al., 2006), RGD-binding

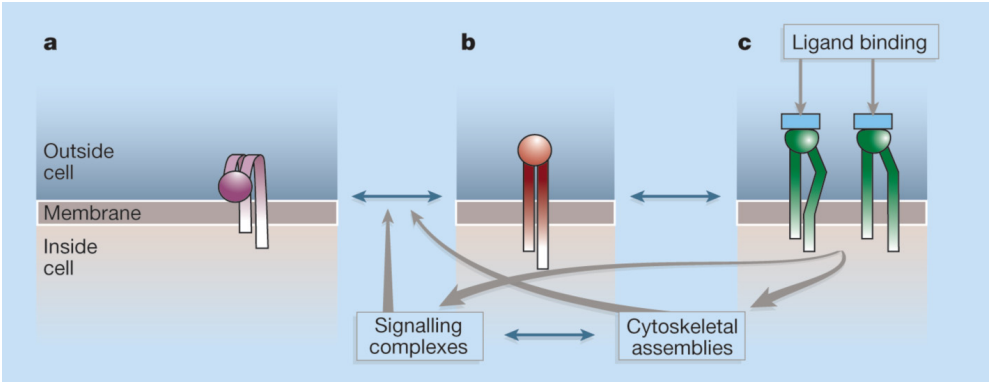

Figure 1. Activation states of integrins. Integrins exist in various conformational states (a-c) having different ligand binding properties. The bent/closed form (a) has a low affinity for ligands, whereas the straight/open one (b) is a high affinity form. Ligand binding to the extracellular region induces a series of conformational changes, which are mediated through the membrane to the intracellular region of the integrins leading to the dissociation of the cytoplasmic domains of the α and β subunits and thus to changes in the signaling pathways (c). This is a bidirectional signaling system, meaning that the binding of intracellular signaling molecules to the cytoplasmic domains of integrins induces a series of conformational changes, which also affect the structure of the extracellular region of the integrins and leads to changes in their ligand binding properties. *(Mould & Humphries, 2004a, Nature 432, 27-28. Reprinted with permission from Macmillan Publishers Ltd.).*

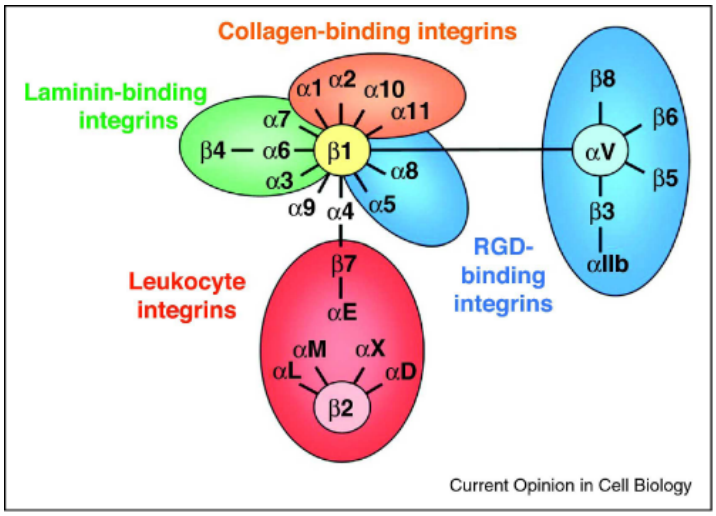

Figure 2. The family of integrin receptors. The 24 mammalian heterodimeric integrins are composed of various combinations of eighteen different α and eight β subunits. The α subunits of collagen receptors and leukocyte-specific receptors have an extra domain called the αI domain (see the text). *(Margadant et al., 2011, Curr. Opin. Cell Biol. 23, 607-614. Reprinted with permission from Elsevier).*

integrins which bind ligands with an Arg-Gly-Asp recognition motif (Pytela et al., 1986), leukocyte specific integrins (section 1.1.3.) and a group including $\alpha_9\beta_1$ and $\alpha_4\beta_1$ integrins (Vandenberg, 2008; Gupta & Vlahakis, 2010) (Figure 2). Most integrins are not strictly ligand specific, but instead they are able to bind different kinds of ECM ligands, and vice versa many ECM ligands are able to bind to different integrins (Hynes, 2004; Meves et al., 2009). Integrins differ from each other also based on their intracellular binding partners and on the consequences their activation has inside the cell. Integrins can also be divided into two groups depending on whether they include an extra domain, called the αI domain in the extracellular region of the α subunit. Half of the mammalian integrin α subunits have the αI domain, which is responsible for the ECM ligand binding in that group of integrins (Humphries, 2000; Heino, 2000; Hynes, 2002; Moser et al, 2009; Xie et al., 2010) (Figure 2).

Even though integrins exist in all tissues and cells in multicellular organism, no cell type expresses all the different integrins of that species. Each cell has under certain conditions a specific, dynamic repertoire of integrins, which changes with the developmental age of the cell and/or when the microenvironmental conditions of the cells are otherwise altered (Barczyk et al., 2010). The expression of integrins is regulated by the extracellular milieu of the cell so that the cell produces integrins which can interact with the ECM (Humphries et al., 2006; Kim et al., 2011). Cells, which fail to bind to the ECM, will die as a result of apoptosis. Furthermore, the expression of integrins in cells which do not have the corresponding ECM ligand in their extracellular matrix space will initiate programmed cell death (Frisch & Ruoslahti, 1997; Kuphal et al., 2005; Stupack, 2005; Mayadas & Cullere, 2005). This kind of apoptosis induced by the absence of the receptor's ECM ligand is called anoikis, which means homelessness (Frisch & Francis, 1994). As shown by the phenotypes of

knockout mice, integrins play diverse and important role in many biological processes, such as development, immune responses, leukocyte traffic, inflammation, hemostasis, bone remodeling, and angiogenesis. Therefore, integrins are also involved in many human diseases, such as cancers, genetic and autoimmune diseases, and others (Hynes, 2002; Barczyk et al., 2010; Shattil et al., 2010; Desgrosellier & Cheresh, 2010). Accordingly, plenty of effort has been invested in studies aiming at developing integrin antagonists for the treatment of cardiovascular, inflammatory and metastatic diseases (Shimaoka & Springer, 2003; Mould & Humphries, 2004b; Lebwohl et al., 2003; Kuphal et al., 2005; Mulgrew et al., 2006; Käpylä et al., 2007; Paolillo et al., 2009; Heino & Käpylä, 2009; Miller et al., 2009; Nissinen et al., 2010; Koivunen et al., 2011).

1.1.2. Collagen receptor integrins and their ligands: collagens and laminins

Collagen receptor integrins. The research described in this Ph. D. thesis mainly deals with integrins which bind collagens (Tulla et al., 2008; Lahti et al, 2011; Käpylä et al., 2007). Humans have four collagen receptor integrins, each of which has the same β subunit (β_1), but a different α subunit (α_1, α_2, α_{10} and α_{11}) (Heino, 2000; Popova et al., 2007) (Figure 2). In addition to collagens, all collagen receptor integrins also bind laminins (Tulla et al., 2008). Furthermore, $\alpha_2\beta_1$ integrin binds tenascin C (Sriramarao et al., 1993), chondoadherin (Camper et al., 1997), matrix metalloproteinase I (Dumin et al., 2001), adhesion receptor E-cadherin (Whittard et al., 2002), proteoglycans (Guidetti et al., 2002; Bix et al., 2004) and collectins (Zutter & Edelson, 2007). Human collagen receptor integrins can recognize their collagenous ligands in ways dependent or independent of the so-called triple helical GFOGER sequence (O denotes hydroxyproline) (Knight et al., 2000; Emsley et al., 2000; Käpylä et al., 2004; Nymalm et al., 2004)(see section 1.3.2).

Integrin $\alpha_1\beta_1$ is abundant in smooth muscle cells and $\alpha_2\beta_1$ is the major collagen receptor in epithelial cells and platelets. Many cell types, including fibroblasts, osteoblasts, chondrocytes, endothelial cells, and lymphocytes may express both of these receptors at the same time (Heino, 2000; White et al., 2004; Popova et al., 2007). Integrin $\alpha_{10}\beta_1$ is mainly found in cartilage and $\alpha_{11}\beta_1$ in many mesenchymal tissues (Camper et al., 1998, 2001; Tiger et al., 2001; Heino et al., 2009). Owing to the small structural differences in their α subunits, the four collagen receptor integrins have different specificities for their extracellular and intracellular ligands, and their activation leads to different physiological consequences. For example, the activation of $\alpha_1\beta_1$ integrin by collagen can induce cell proliferation and inhibit collagen synthesis, whereas the activation of $\alpha_2\beta_1$ integrin by collagen stimulates both the synthesis of collagen and collagenase. $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are also involved in cancer (White et al., 2004; Heino, 2007). $\alpha_{11}\beta_1$ may partially replace $\alpha_2\beta_1$ integrin as the main collagen receptor in prostate cancer cells having effects on cell migration (Mirtti et al., 2006). The activation of $\alpha_{11}\beta_1$ transcription has been observed also at the messenger RNA (mRNA) level in melanomas (Vuoristo et al., 2007) and non-small-cell lung cancer (Wang et al., 2002; Zhu et al., 2007a). Knockout-mouse experiments on individual collagen receptor integrin α subunits have shown phenotypes milder than those caused by deletions of integrins α_3-α_8 and α_V subunits, for example, suggesting functional redundancy between collagen-binding integrins (Hynes, 2002; Heino et al., 2009; Popova et al., 2007; Leitinger & Hohenester, 2007; Leitinger, 2011).

In addition to integrins, members of a structurally diverse group of transmembrane receptors, such as discoidin domain receptors, glycoprotein IV, and leukocyte-associated immunoglobulin-like receptor-1, for example, can bind collagens (Leitinger & Hohenester, 2007; Heino et al., 2009; Leitinger, 2011).

Collagens. Collagen is the most abundant protein in animals. In humans, collagen comprises about 1/3 of total protein. Each tissue has a specific set of collagens. A partial listing of the collagen types found in different tissues is given by Shoulders & Raines (2009). Collagen is an important component of the ECM having a key role in the maintenance of the structure of various tissues. Furthermore, collagens are involved in cell adhesion, chemotaxis and migration. The interactions of collagens with cells regulate tissue remodeling, differentiation, morphogenesis and wound healing (Myllyharju & Kivirikko, 2004). All collagens are composed of three subunits forming a triple helical structure, which is important for interactions with other proteins including cell surface receptors (Leitinger, 2011). There are both homo- and heterotrimeric collagens. In vertebrates 28 collagen types (I-XXVIII) composed of 46 different subunits have been identified. There are also many other proteins which contain collagenous domains but are not considered as collagens (Myllyharju & Kivirikko, 2004; Heino et al., 2009; Shoulders & Raines, 2009). At least part of the primary structure of the collagen subunit is monotonous containing repeating sequences of Gly-X-Y, where X and Y are frequently proline and hydroxyproline, respectively. Gly is important for the formation of the triple helix and hydroxyproline is important for its stability (Myllyharju & Kivirikko, 2004). Collagens are classified based on the structures they form and the structural roles they have. Accordingly, vertebrates have fibrillar collagens (I-III, V, XI, XIV, XXVII), network-forming collagens (IV, VI, VIII, X), fibrils-anchoring collagen (VII), fibril-associated collagens with interrupted triple helices (FACIT; IX, XII, XIV, XVI, XIX-XXII, XXVI), membrane-associated collagens with interrupted triple helices (MACIT; XIII, XVII, XXIII, XXV), and MULTIPLEXIN collagens which contain multiple triple-helical domains with interruptions (XV, XVIII) (Shoulders & Raines, 2009). The recently discovered

collagen type XXVIII is an αI domain-containing protein with an imperfect collagenous domain (Veit et al., 2006; Shoulders & Raines, 2009).

Laminins. Laminins are multidomain, heterotrimeric proteins composed of three polypeptide chains (α, β, γ) held together by disulfide bonds (Colognato & Yurchenco, 2000). Five α chains, three β chains and three γ chains are known for mouse and human. These eleven subunits can form at least 16 different α-β-γ combinations (Aumailley et al., 2005). Laminins have T- or cross-shaped structures with two or three short arms, respectively, and one long arm. Each short arm includes part of one polypeptide chain, whereas in the long arm parts of all three subunits are twisted together to form a coiled-coil domain which is typical for laminins (Colognato & Yurchenco, 2000; Aumailley et al., 2005). Laminins are found in the basal lamina where they form sheetlike networks with other ECM-proteins. The composition of the basal lamina network varies in different tissues, but it typically includes the glycoproteins laminin, collagen type IV and nidogen (also called entactin), as well as the proteoglycan perlecan. Nidogen links together the networks of laminin and collagen IV. The basal lamina network is attached to the plasma membrane by the transmembrane collagen and laminin receptors, integrins and dystroglycan (Colognato & Yurchenco, 2000).

1.1.3. Leukocyte specific integrins

Leukocyte specific integrins were studied in the final paper of this Ph.D. thesis (Paper IV; Lahti et al., 2012). There are five leukocyte specific integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_E\beta_7$)(Figure 2), though at least 12 different integrins are expressed in various types of leukocytes and platelets (Luo et al., 2007; Zhang & Wang, 2012). Leukocyte specific integrins are important for the immunoresponse that activates white blood cells. Leukocytes use these

adhesion receptors to migrate through the endothelial cells to invade tissues that show signs of inflammation (Rose et al., 2007; Zhang & Wang, 2012). Ligands for leukocyte specific integrins include, for example, ICAM-1 (Intercellular cell adhesion molecule 1), VCAM-1 (Vascular cell adhesion molecule 1), E-cadherin, fibrinogen, vitronectin, inactive complement factors (iC3b, iC4b) and factor X (Humphries, 2000; Humphries et al. 2006; Barczyk et al., 2010; Margadant, 2011; Zhang & Wang, 2012). However, some leukocyte specific integrins, for example complement receptors, $\alpha_M\beta_2$ (CR3) and $\alpha_X\beta_2$ (CR4), as well as $\alpha_L\beta_2$ also bind collagens (Lahti et al., 2012; Zhang & Wang, 2012). Structural and functional similarities between collagen receptor integrins and leukocyte specific integrins pose a big challenge for structure-based drug design. Selectivity is a key issue: the drugs against collagen receptor integrins should not block leukocytes integrins, and vice versa.

1.2. THE STRUCTURE OF INTEGRINS

By the mid of 1970's plenty of evidence had been gathered indicating that extracellular matrix proteins and the intracellular cytoskeleton were somehow linked together (Hynes, 2004). Hynes (1976) proposed that there are integral membrane proteins that connect an extracellular matrix protein, fibronectin, to the actin microfilaments of the cytoskeleton. By the mid of 1980's cDNAs encoding receptor proteins had been cloned and their nucleotide sequences determined, revealing the corresponding amino acid sequences. By comparing the structural and functional properties of twelve different plasma membrane receptors Hynes and his collaborators found these proteins to be highly similar and thus proposed that they form a family of plasma membrane receptors which they called integrins (Tamkun et al., 1986; Hynes, 1987). In 1987 the following was known about the structure of integrins: Integrins are heterodimeric, transmembrane glycoproteins composed of an α and a β subunit, which are connected by non-covalent interactions. Both subunits have a short C-terminal

cytoplasmic tail, one transmembrane domain, and a long N-terminal extracellular region. The primary structure of the β subunit, including four cysteine-rich repeat sequences of 40 amino acids, suggested that this subunit has several disulfide bonds. Some of the α subunits are posttranslationally cleaved into two parts forming heavy and light chains, which are connected to each other by disulfide bonds (Hynes, 1987).

The α and β subunits are not homologous and the sizes of integrin subunits vary: α subunits are in total 1025-1188 residues long and β subunits 769-799 residues long with the exception of β_4 which has 1875 amino acid residues. Three α subunits and four β subunits have splice variants (α_3/2, α_6/2, α_7/4, β_1/4, β_3/3, β_4/5, and β_5/2; the number in the denominator indicates the number of splice variants) (Barzyk et al., 2010). The extracellular regions of integrins are long, about 700 – 1100 residues (the extracellular regions of α subunits are > 940 residues and β subunits > 640 residues; Takagi & Springer, 2002), and the cytoplasmic regions are short, only about 15-50 amino acids (Humphries, 2002; Gahmberg et al., 2009). In this respect the β_4 subunit (Figure 1) differs from all the other integrin subunits as its intracellular domain is very long, about 1000 residues. Because of this the intracellular region of the β_4 subunit is able to bind directly to the intermediate filaments of the cytoskeleton whereas other integrins with short cytoplasmic domains need various mediator proteins for binding to the actin microfilament cytoskeleton (van der Flier & Sonnenberg, 2001; Hynes, 2002; Moser et al., 2009; Kim et al., 2011).

A three-dimensional structure of an intact integrin embedded in a membrane has not yet been solved. Instead, 3D structures of the extracellular region (ectodomain) are available for three integrins, two of which lack an αI domain [αI-less integrins, $\alpha_v\beta_3$ (Xiong et. al., 2001, 2002, 2009) and $\alpha_{IIb}\beta_3$ (Zhu et al.,

2008)] and one with an αI domain ($\alpha_x\beta_2$, Xie et al., 2010). Furthermore, 3D structures have been solved for the headpiece regions (see section 1.2.1.) of $\alpha_{IIb}\beta_3$ (Xiao et al., 2004; Springer et al., 2008; Zhu et al. 2010), $\alpha_5\beta_1$ (Nagae et al., 2012) and $\alpha_4\beta_7$ (Yu et al., 2012) integrins, for several integrin αI domains [α_L (Qu & Leahy, 1995; Shimaoka et al., 2003), α_M (Lee et al., 1995; Li et al., 1998), α_X (Vorup-Jensen et al., 2003), α_1 (Nolte et al., 1999; Rich et al., 1999; Nymalm et al., 2004), α_2 (Emsley et al., 1997, 2000)], and for the transmembrane domain of $\alpha_{II}\beta_3$ composed of two α-helices (Lau et al., 2009).

Interestingly, all the 3D structures of the extracellular region are in the bent conformation (Figure 1) irrespective of the presence or absence of ligands. However, bent and extended conformations have been seen by electron microscopy for both αI-less ($\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$) integrins and for integrins including an αI domain ($\alpha_x\beta_2$, $\alpha_L\beta_2$) (Takagi & Springer, 2002; Zhu et al., 2008; Chen et al., 2010, 2012; Xie et al., 2010; Springer & Dustin, 2012). The structurally simple transmembrane domain and the short cytoplasmic tails have a key role in integrin activation and are described in section 1.5. Next, the 3D structures of the integrin extracellular region and αI domains are briefly described.

1.2.1. The structure and domains of the integrin extracellular region

The extracellular regions of both α and β subunits of integrins are large consisting of several domains. The schematic representation in Figure 3 shows the locations of these domains in the primary structure and in the tertiary structure of the bent integrin molecule. Figure 4A shows the first solved three-dimensional structure of the integrin extracellular region, that of $\alpha_V\beta_3$. Integrins have a spherical headpiece region responsible for the ligand binding and long leg/arm region penetrating the plasma membrane. αI-less integrins have 12

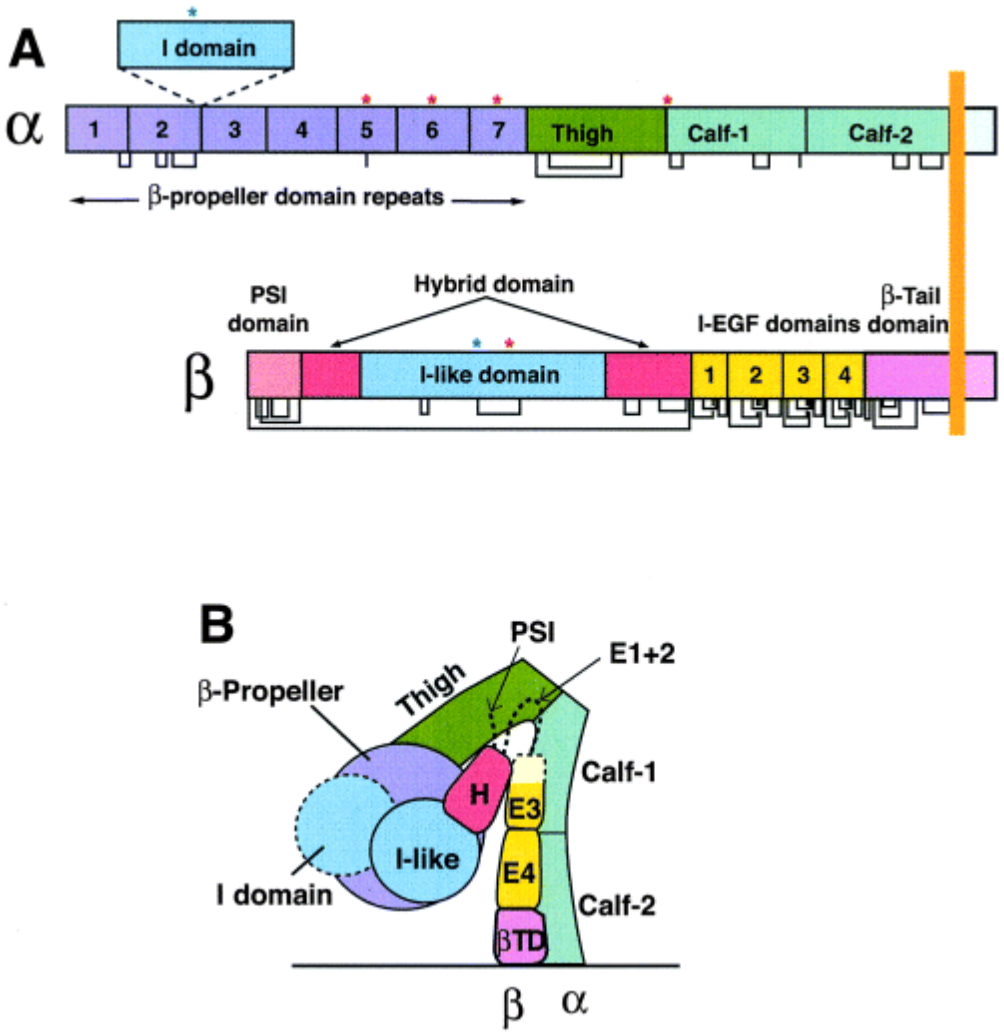

Figure 3. Schematic presentation of the location of the domains in the primary and bent tertiary structure of the intergin α and β subunits. A) Half of the mammalian integrin α subunits (Figure 2) include an αI domain, which is the ligand binding site in those integrins. The location of the αI domain is shown by a dashed line. The asterisks show the binding sites of Mg^{2+} (blue) and Ca^{2+} (red) ions. The lines below the domain boxes indicate disulfide bonds. B) This model has been built based on the crystal structure of $\alpha_V\beta_3$ integrin (Xiong et al., 2001). In this model the location of the αI-domain is shown even though $\alpha_V\beta_3$ integrin lacks this domain. The 3D structures of PSI and EGF1-2 domains could not be accurately determined and so they are presented in Figure B by dashed lines. The domains are coloured in the same way in Figures A and B. *(Springer, 2002, Curr. Opin. Struct. Biol. 12, 802-813. Reprinted with permission from Elsevier).*

extracellular domains (Figures 4A and B). Their spherical headpiece region is composed of three domains, the β-propeller domain of the α subunit as well as the βA domain and the immunoglobulin-like hybrid domain of the β subunit (Figures 4A and B). The β-propeller domain mainly consists of β sheets and contains seven ~ 60 residue repeats (Figure 3A). The βA domain structurally closely resembles the I domain of the α subunit, αI, and is thus also called the I-like (βI) domain (Figure 3). Hereafter, this domain is referred to as βI in the text. Half of the mammalian integrin α subunits include a ~200 residue-long αI domain (see section 1.2.2.), which is located in the headpiece region of the molecule (Figure 3B).

The leg/arm region of the α subunit consists of three domains, thigh, calf1, and calf2, which are mainly β-sheet structures. The leg-region of the β subunit includes six domains, the PSI domain (named based on three proteins, Plextrin, Semaphorin and Integrins, which all have this domain; Bork et al., 1999), four epidermal growth factor–like, cysteine-rich domains (EGF1-4) and the β-tail domain (βTD) (Figure 4). Xiong et al. (2001) was not able to accurately solve the linker 1 region of the α subunit nor the linkers 2-3 or the domains PSI and EGF1-2 of the β subunit suggesting that they are flexible parts of the protein.

The domains of the α subunit extracellular region are in the same order in the primary and tertiary structure, the N-terminus is in the headpiece β-propeller domain and the C-terminus in the calf2 domain of the leg-region. In integrins which have an αI domain, the αI domain has been inserted between the second and third repeat elements of the β-propeller (Figure 3A). In this respect the structure of the β subunit is more complicated than that of the α subunit since

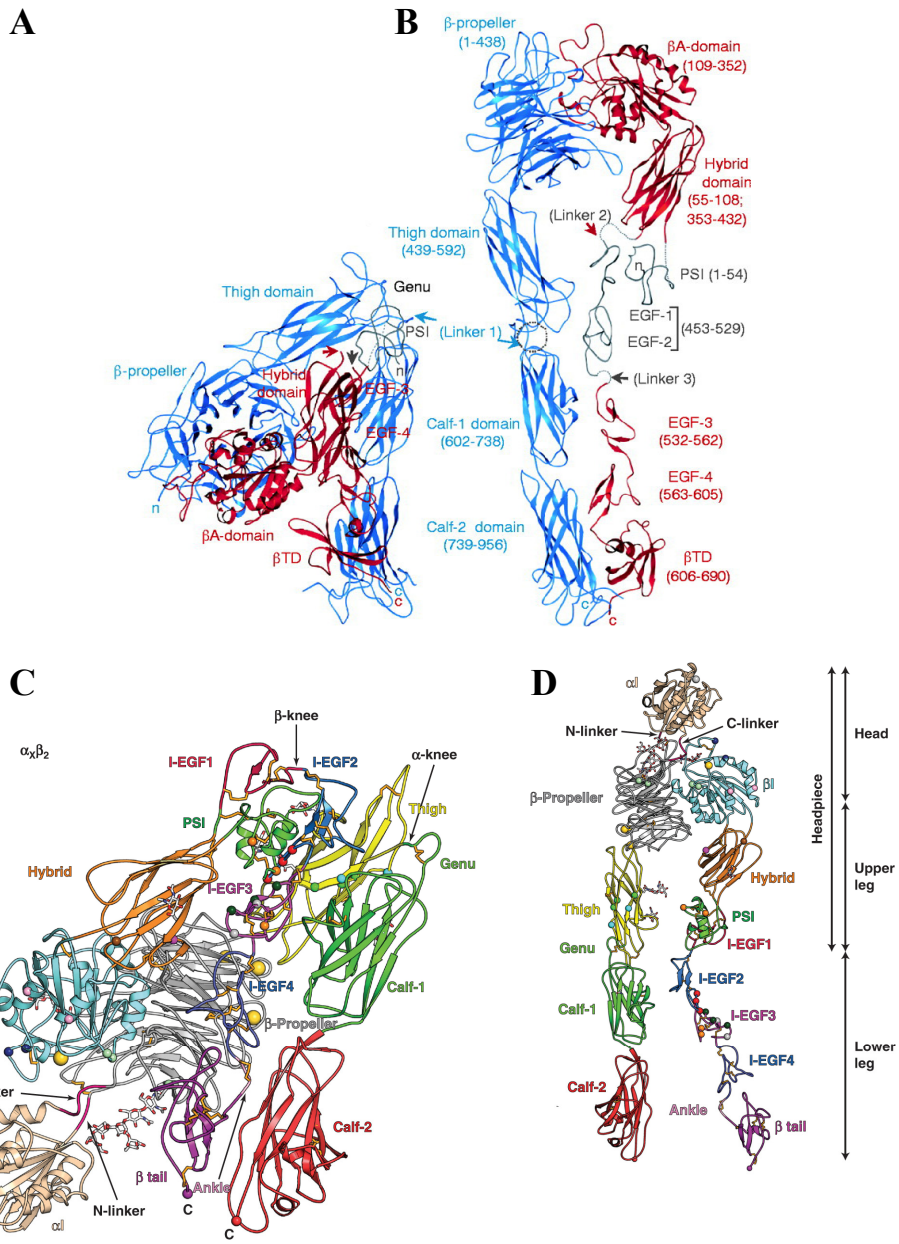

Figure 4. Three-dimensional structure of the extracellular regions of $\alpha_V\beta_3$ and $\alpha_x\beta_2$ integrins. The 3D structures of the bent forms of $\alpha_V\beta_3$ (A) and $\alpha_x\beta_2$ (C) were solved by X-ray analysis. The sraight/extended conformations of $\alpha_V\beta_3$ (B) and $\alpha_x\beta_2$ (D) were modelled based on the bent structures. The numbers in brackets in Figure 4B refer to the amino acid sequences clearly showing the locations of the domains within the primary structure in both subunits. The α subunit is shown in blue and β subunit in red in Figures A and B. *(A and B: Xiong et al., 2001, Science 294, 339-345; C and D: Xie et al., 2010, EMBO J. 29, 666-679. Reprinted with permission from the American Association for the Advancement of Science* (*AAAS*) *and Macmillan Publishers Ltd., respectively).*

the headpiece domains (βI and hybrid) are not at the N-terminus: the PSI domain is the N-terminal domain of the β subunit. A hybrid-domain has been inserted between the PSI and EGF1 domains, and the βI domain inserted within the hybrid domain (Figures 3 and 4).

The extracellular region of integrins is large and thus there are numerous interactions between and within the subunits. These inter- and intramolecular interactions have been reviewed in detail by Arnaout (2002) and are only briefly described here. Based on the 3D structure of $\alpha_V\beta_3$ integrin the most significant intermolecular contact is between the β-propeller and βI domains (Figure 4B). There are also plenty of other intermolecular contacts, such as β-propeller:EGF3, β-propeller:EGF4, thigh:EGF3, calf2:EGF4, and calf2:βTD. However, these contact areas are quite small and thus their interactions are rather weak. Accordingly, they may not significantly affect the intact integrin bound to the plasma membrane (Xiong et al., 2001). The following observations reviewed by Arnaout (2002) support the idea that the headpieces of the α and β subunits and especially the contact between the β-propeller and the βI domain are mainly responsible for the heterodimeric structure of integrins: A) C-terminal deletions of α and β subunits lacking leg parts but including headpieces are still able to form heterodimers with each other. These types of deletions were produced by proteolytic digestions. B) Most of the mutations preventing the formation of the heterodimeric structure and thus resulting in the dysfunction of β_2 and β_3 integrins, are found in the βI domain. C) The βI domain will fold into its native conformation after it has come into contact with the β-propeller domain; i.e., the intermolecular β-propeller:βI contacts are necessary for the proper folding of the βI domain. The contacts between subunits are more extensive in the bent conformation than in the extended conformation. The formation of heterodimeric integrins occurs inside

the cell before the protein is transported to the cell surface (Humphries, 2000), i.e., free α and β subunits do not exist at the cell surface (Barczyk et al., 2010).

The overall 3D structures and subunit contacts of the bent extracellular regions of integrins $\alpha_{IIb}\beta_3$ (Zhu et al., 2008) and $\alpha_x\beta_2$ (Xie et al., 2010) are quite similar to that of $\alpha_V\beta_3$ (Xiong et. al., 2001, 2002) except the terminal domains calf2 and β-TD of the α- and β-legs, respectively, are oriented differently in the αI-less integrins $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ than in $\alpha_x\beta_2$, which has an αI domain (Figure 4).

1.2.2. The structure of the αI domain

The αI domain, also called the von Willebrand A (VWA) domain, is present in numerous proteins including integrins. VWA domains are generally found in multiprotein complexes and seem to be responsible for protein:protein interactions. Divalent metal ions are essential for many VWA domains (Whittaker & Hynes, 2002). The αI domain folds independently into a native, functional conformation. Accordingly, it is easy to produce and purify this domain as a soluble protein for structural and functional analyses. For this reason several αI domains have been characterized in a detailed way (for reviews see Arnaout, 2002; Takagi & Springer, 2002; Luo et al., 2007); for example, three-dimensional structures have been solved for the αI domains of α_L (Qu & Leahy, 1995; Shimaoka et al., 2003), α_M (Lee et al., 1995; Li et al., 1998), α_X (Vorup-Jensen et al., 2003), α_1 (Nolte et al., 1999; Rich et al., 1999; Nymalm et al., 2004; Lahti et al., 2011) and α_2 (Emsley et al., 1997, 2000).

For the sake of comparison Figure 5 shows the 3D structures of the α_2I, α_LI and α_MI domains and the VWA3 domain. As seen in Figure 5, all these four structures are very similar to each other and they all include the classical Rossmann fold structure (Rossmann et al., 1974). The core of the Rossmann

fold in the αI domains contains 5 parallel and 1 short antiparallel β sheet, surrounded by 6 α helices. In addition to these 6 α helices found in all αI domains, some αI domains also have short α helices, the number and size of which differ between the αI domains (Takagi & Springer, 2002). In the topological structure each α helix is followed by a β sheet. The Rossmann fold is present in a large variety of proteins and enzymes, which normally bind dinucleotides (Gherardini et al., 2010).

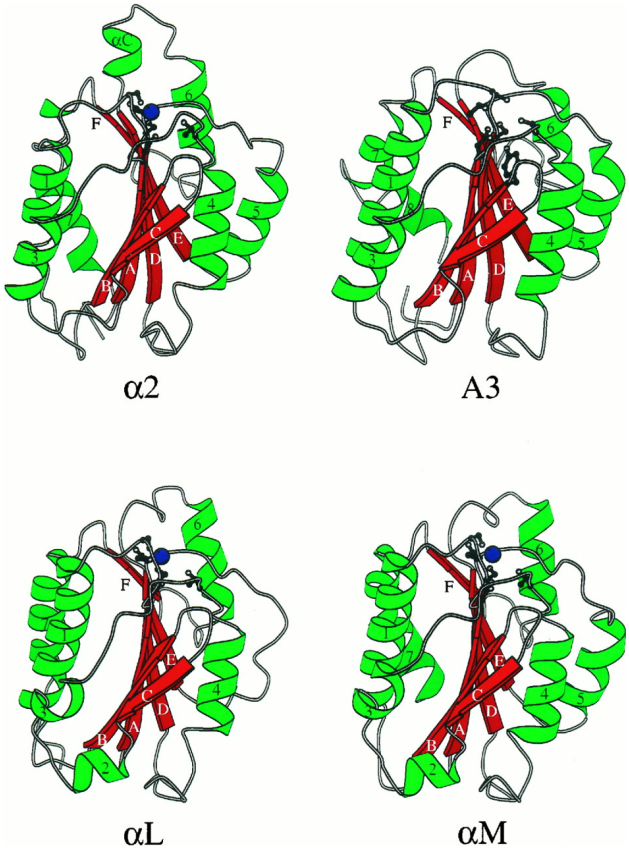

Figure 5. Three-dimensional structures of the α_2I, α_LI, α_MI and vWF-A3 domains. vWF-A3 is one of the three VWA domains of the *von Willebrand Factor* (vWF). vWF is a vertebrate extracellular matrix protein mediating adhesions between platelets and fibrillar collagen (Sadler, 1998). The divalent metal ion bound to the αI domain (shown in blue circle) has a significant role in mediating ligand binding to integrins. *(Emsley et al., 1997, J. Biol. Chem 272, 28512-28517. Reprinted with permission from The American Society for Biochemistry and Molecular Biology).*

1.3. THE METAL ION AND LIGAND BINDING SITES OF THE EXTRACELLULAR REGION OF INTEGRINS

1.3.1. Binding sites of metal ions

Divalent metal ions are essential for integrins. There are several metal ion binding sites in integrins each having a different specificity and affinity. Mn^{2+} and Mg^{2+} stimulate and Ca^{2+} inhibits ligand binding. In the presence of Mn^{2+} ligand binding is stronger than with Mg^{2+}. Equilibrium dialysis measurements suggested that integrins have 4-5 binding sites for divalent metal ions (Humphries, 2000). However, in the 3D structure of the extracellular region of the ligand-free $\alpha_v\beta_3$ integrin 6 metal ion binding sites were identified (Xiong et al., 2001), 4 in the α subunit and 2 in the β subunit (Figure 3A), whereas in the ligand-bound form one additional metal ion binding site was identified in each subunit (Xiong et al., 2002). As far as I know, the physiological roles of these eight metal ion binding sites are still unclear. It has been proposed that at least some of them would be important for stabilizing the structure of integrins (Humphies et al., 2003). Of these eight metal ion binding sites three located in the βI domain have been studied in a detail. These three sites are called MIDAS (Metal ion-dependent adhesion site), ADMIDAS (Adjacent to MIDAS) and LIMBS (Ligand-associated metal binding site) (Figures 6 & 7). ADMIDAS and LIMBS are important for the function of integrins. MIDAS, which binds either Mn^{2+} or Mg^{2+}, is located between these two sites.

In integrins lacking an αI domain, the MIDAS metal ion of the βI domain is directly involved in ligand binding and generally interacts either with aspartate or glutamate depending on the ligand. ADMIDAS and LIMBS metal ions regulate ligand binding. Studies on the ADMIDAS site of various integrins have given somewhat contradictory results. It seems that the Ca^{2+} ion is the

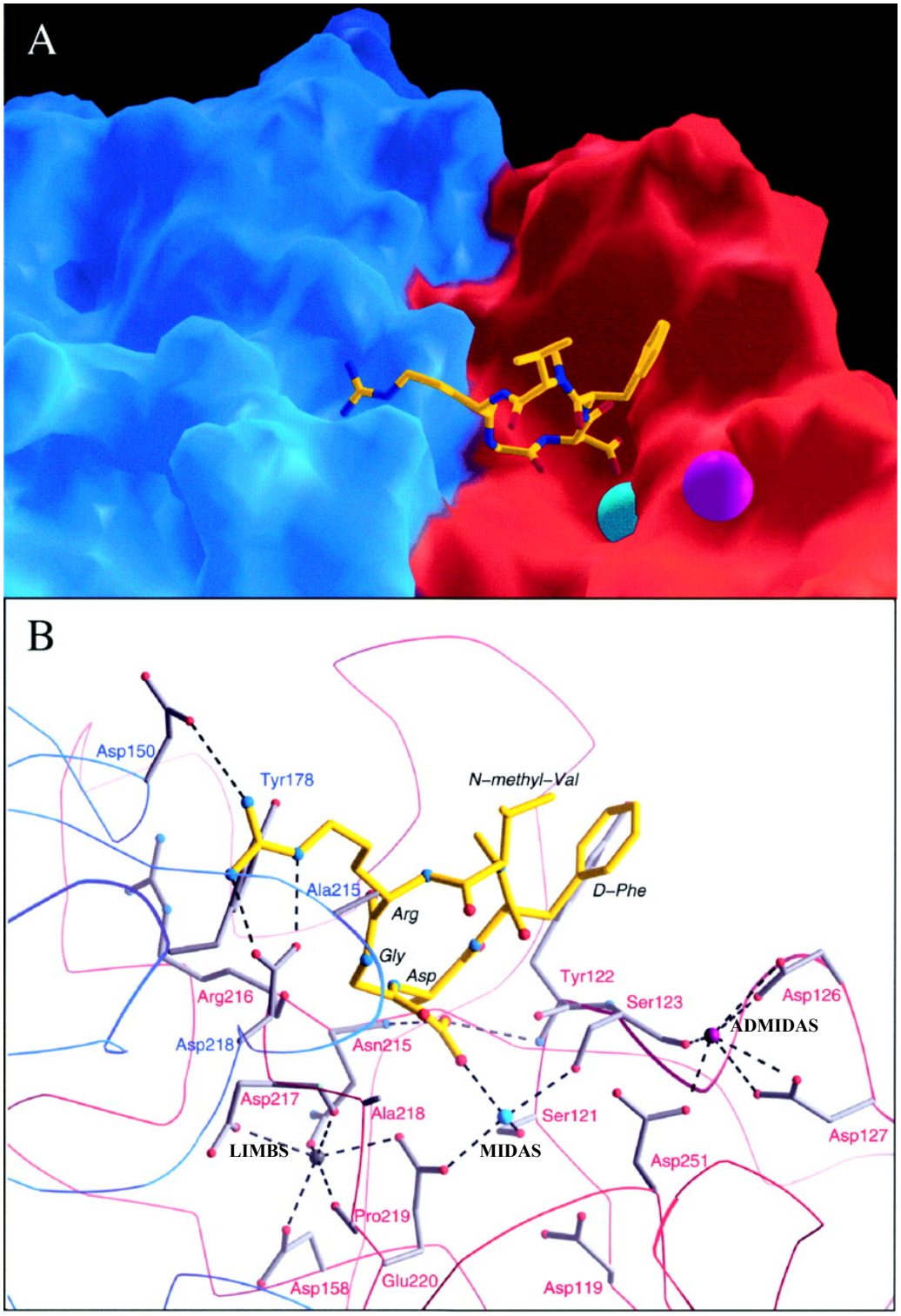
A
B
Asp150
Tyr178
N–methyl–Val
Ala215
Arg
D–Phe
Gly
Asp
Tyr122
Ser123
Asp126
ADMIDAS
Arg216
Asp218
Asn215
Asp217
Ala218
Ser121
Asp127
LIMBS
MIDAS
Asp251
Pro219
Asp158
Glu220
Asp119

Figure 6. Binding site of a small-molecular, circular RGDF-peptide ligand in $\alpha_V\beta_3$ integrin. A) The β-*propeller* domain of the α_V subunit is shown in blue and the βI domain of the β_3 subunit in red. Mn^{2+} ions at the MIDAS and ADMIDAS sites are shown by blue and violet balls. B) Integrin:ligand interactions. The amino acid residues of the α_V subunit interacting with the ligand are shown in blue and the residues of the β_3 subunit interacting with the ligand are shown in red. Hydrogen bonds and salt bridges are indicated by dashed lines. There are three Mn^{2+} ions close to the ligand binding site. The metal ions at the MIDAS, ADMIDAS and LIMBS sites are shown by blue, red and grey balls, respectively. *(Xiong et al., 2002, Science 296, 151-155. Reprinted with permission from AAAS).*

physiological effector in both the LIMBS and ADMIDAS sites and that Ca^{2+} in the LIMBS site stimulates, and in ADMIDAS inhibits ligand binding (Mould & Humphries, 2004b; Arnaout et al., 2005; Luo et al., 2007; Barczyk et al., 2010). However, in the case of $\alpha_{IIb}\beta_3$ integrin Ca^{2+} at the ADMIDAS site together with Mg^{2+} at the MIDAS site have been shown to be involved in ligand binding (Springer et al., 2008). Based on the 3D structure of the extracellular region of $\alpha_V\beta_3$ integrin, metal ion binding to the MIDAS and ADMIDAS sites is independent of ligand binding, whereas a metal ion is seen at the LIMBS site only in the presence of a ligand (Xiong et al., 2001; Xiong et al, 2002). Integrins with an αI domain have the three metal ion binding sites mentioned above and an additional metal ion binding site in the αI domain, which is also called the MIDAS site. In these integrins the metal ion at the MIDAS site of the αI domain is essential for ligand binding.

1.3.2. Binding sites of ligands

Before the 3D structure of a ligand complex of an integrin extracellular region was solved, the following kinds of attempts were made to identify the ligand binding sites of integrins: a) ligands were tested for binding with different parts of the extracellular ectodomain of integrins and with chimeric integrins; b) ligands were covalently bound to integrins and then the residues covalently bound to the ligands were identified; c) ligand binding sites were mapped with

monoclonal antibodies; d) site-specific mutagenesis was used to identify residues responsible for ligand binding. In these studies reviewed by Humphries (2000), the conclusions reached were in good agreement with the 3D structures solved afterwards, where the ligand binding sites could be seen in detail.

A small-molecular ligand including the RGD-recognition sequence (Pytela et al., 1985, 1986) binds to the β-propeller domain of the α subunit and the βI domain of the β subunit in $\alpha_V\beta_3$ integrin; i.e., the ligand binds to the subunit interface interacting with amino acid residues in both subunits (Figure 6). The aspartate in the ligand binds to the Mn^{2+} ion of the MIDAS site in the βI domain and arginine binds by hydrogen bonding to two aspartates (Asp150 and Asp218) in the β-propeller domain (Figure 6B). The binding site of a macromolecular ligand naturally covers a much larger region of the integrin (Xiao et al., 2004) including the top parts of both the β-propeller and the βI domain. The ligand binding site in the β-propeller domain is called the Cap region (Figure 7). 71% of the mutations affecting the binding of a macromolecular fibrinogen ligand are located in the Cap region of $\alpha_{IIb}\beta_3$ integrin and the remaining 29% in the top part of the βI domain and in the small molecular ligand binding site close to the contact region of the β-propeller and the βI domain (Xiao et al., 2004). The schematic presentation of a macromolecular ligand binding to the headpiece of $\alpha_{IIb}\beta_3$ integrin shown in Figure 7 was made based on the 3D structure published by Xiao et al. (2004). Even though the α subunit has a key role in determining ligand specificity (Barczyk et al., 2010) both subunits contribute to ligand binding and selectivity (Hynes, 2002; Nagae et al., 2012).

In integrins with an αI domain the ligand binding site is located in the αI domain. Figure 8 shows the 3D structure of the α_2I domain complexed with a

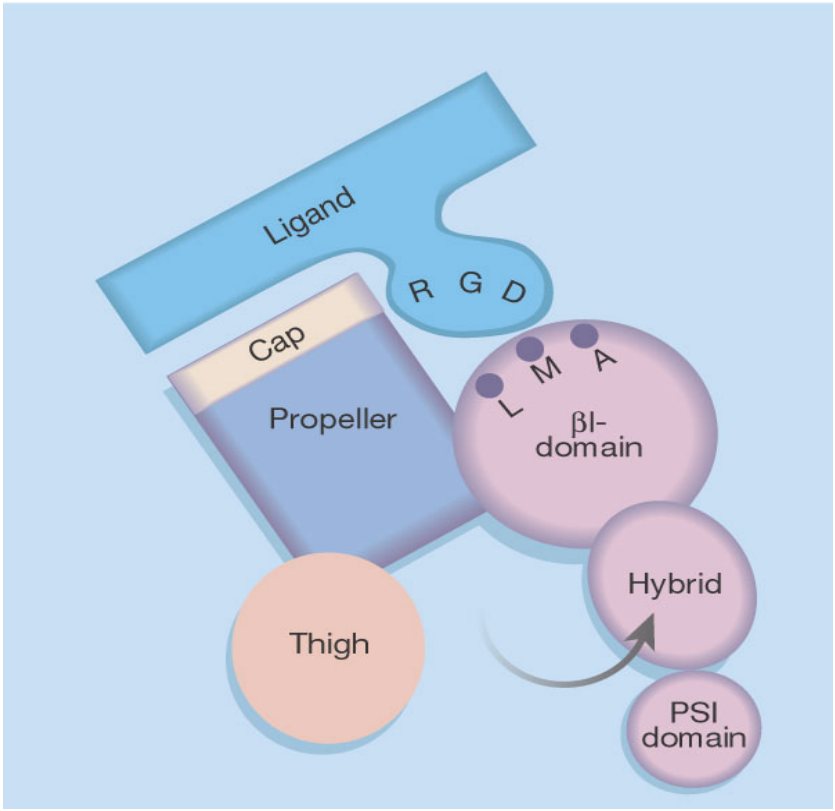

Figure 7. Schematic presentation of a macromolecular ligand binding to an integrin lacking an αI domain. A more detailed presentation of the domain structures is shown in Figure 4. L, M and A are the metal ion binding sites LIMBS, MIDAS and ADMIDAS, respectively. The arrow demonstrates conformational changes induced by ligand binding, including outward movements (swing-out) of hybrid and PSI domains with the result of leg separation. *(Mould & Humphries, 2004a, Nature 432, 27-28. Reprinted with permission from Macmillan Publishers Ltd.).*

synthetic triple helical GFOGER peptide in which O is hydroxyproline. GFOGER (Knight et al., 1998, 2000) is one of the several high affinity binding sites for collagen receptor integrins; others are GROGER, GLOGER, GMOGER, GLOGEN, GAOGER, for example. The distribution of these receptor-binding sites is different in different collagens (Herr & Farndale, 2009; Leitinger, 2011). Collagen receptor integrins have different selectivities towards these binding motifs with $\alpha_2\beta_1$ preferring GFOGER and $\alpha_1\beta_1$ GLOGEN (Hamaia et al., 2012).

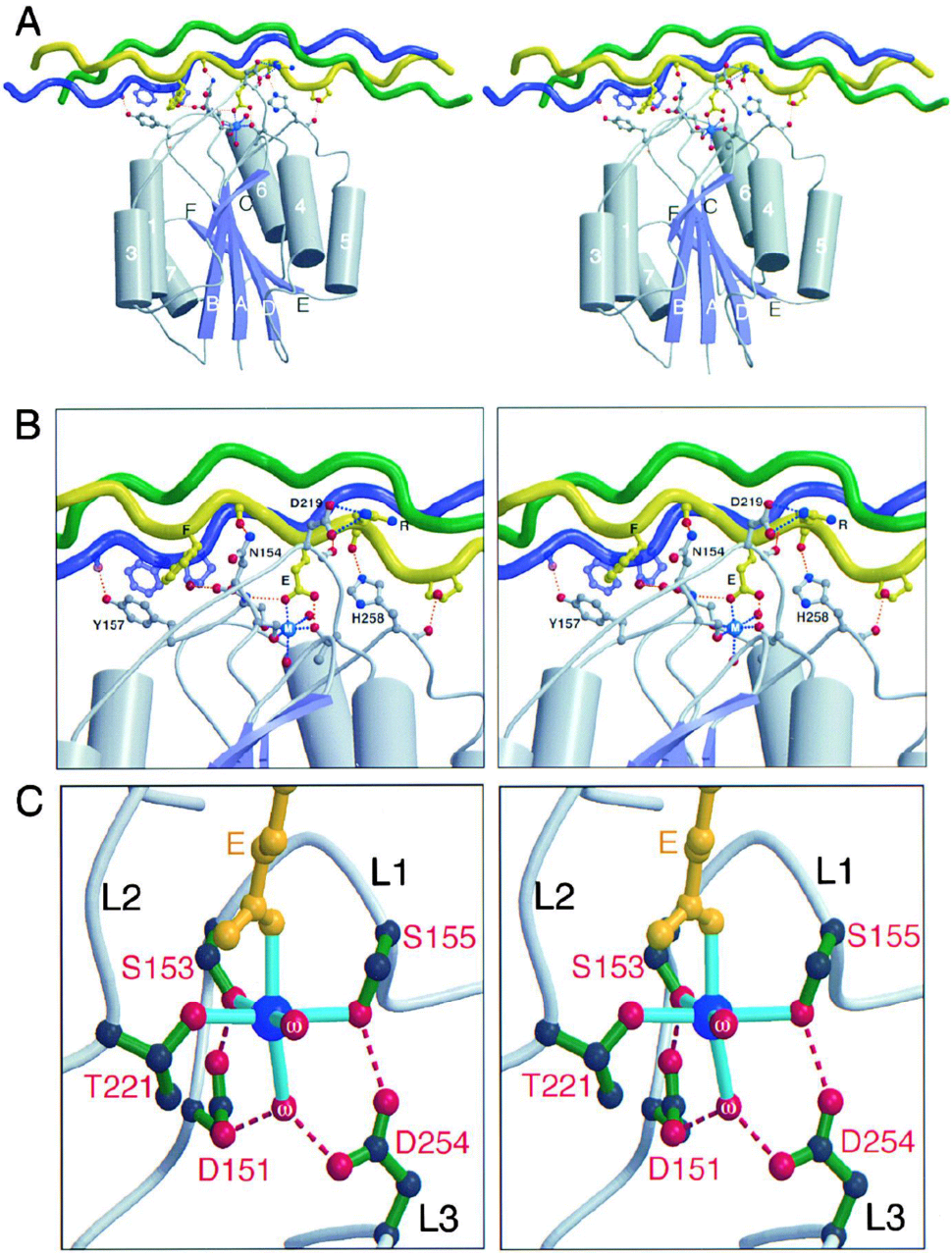

Figure 8. Three-dimensional structure of the α_2I domain:collagen peptide-complex. A) Stereoview of the complex. The three strands of the triple helical collagen peptide are shown in green (leading strand), yellow (middle strand) and blue (trailing strand). B) Enlargement of Figure A showing details of contacts between the αI domain and the collagen ligand. C) Stereoview of the interactions between the MIDAS metal ion and the αI domain. See the text for more details of the interactions. *(Emsley et al., 2000, Cell 101, 47-56. Reprinted with permission from Elsevier).*

The most important interactions between the middle strand of GFOGER (yellow ribbon) and the α_2I domain are: a) the phenylalanine in GFOGER forms a van der Waals contact with asparagine 154 (N154) and glutamine 215 (Q215) of the αI domain; b) the hydroxyproline is hydrogen-bonded to asparagine 154; c) the glutamate is coordinated to the MIDAS metal ion and hydrogen-bonded to threonine 221 (T221); d) the arginine is salt-bridged to aspartate 219 (D219) and hydrogen-bonded to histidine 258 (H258). The interactions between the trailing strand of GFOGER (blue ribbon) and the αI domain are: a) the carbonyl group of the peptide bond preceeding the GFOGER sequence is hydrogen-bonded to tyrosine 157 (Y157) of the αI domain; b) the phenylalanine of the GFOGER peptide connects to leusine 286 (L286) and tyrosine 157 (Y157) through van der Waals interactions; c) the hydroxyproline is hydrogen-bonded to asparagine 154 (N154); d) the arginine shares an ion-bond with glutamate 256 (E256). Some of these interactions are shown in Figure 8. The collagen ribbon shown in green is not connected to the αI domain (Emsley et al., 2000).

The residues important for the binding of the MIDAS metal ion are aspartates 151 and 254 (D151 and D254), serines 153 and 155 (S153 and S155) and threonine 221 (T221) (Figure 8C). In addition, glutamate 256 (E256) binds to the MIDAS metal ion via water, but for clarity, this is not shown in Figure 8C. Site-directed mutagenesis studies have shown that Asp151, Ser153, Thr221 and Asp254 are essential for collagen binding to the integrin α_2I domain (Kamata & Takada, 1994).

Figure 9 shows a surface profile presentation of the αI domain:collagen peptide complex. The following contacts between the αI domain and the middle strand of the collagen fiber are highlighted in Figure 9B: a) the MIDAS metal ion

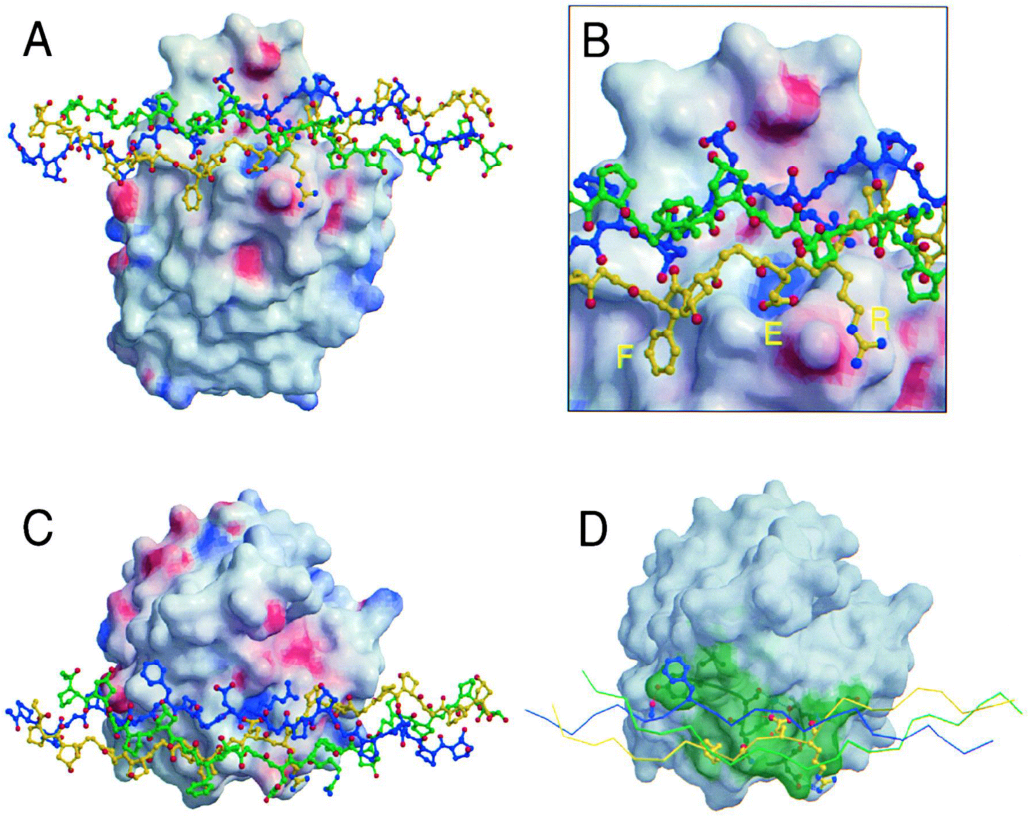

Figure 9. Surface presentation of the α_2I domain:collagen peptide complex. Blue and red colours show positively and negatively charged regions in the αI domain, respectively. The strands of the triple helical collagen peptide are coloured as described in Figure 8. A) The same orientation as in Figure 8. B) Enlargement of Figure A showing some details of the contact surface. C) Top view of the complex. D) The green area is the collagen binding region in the αI domain. *(Emsley et al., 2000, Cell 101, 47-56. Reprinted with permission from Elsevier).*

containing groove with a positive charge, where the negatively charged glutamate of GFOGER-sequence protrudes; b) the phenylalanine of GFOGER sequence is located in the groove of the αI domain surface, and c) the positively charged arginine is salt-bridged to the negatively charged αI domain surface.

The 3D structure of the α_2I domain complexed with a triple helical collagen peptide was a great breakthrough for collagen receptor integrin research

showing the molecular details of contacts between the receptor and the collagen fiber (Emsley et al., 2000). It gave important structural information for research aiming to genetically modify the ligand binding properties of collagen receptor integrins.

Ligand binding is similar in all integrins irrespective of the presence of the αI domain. The carboxyl group of the ligand, which in αI domain-containing integrins is generally glutamate and aspartate in other integrins, coordinates with the MIDAS metal ion. This explains why a divalent metal ion is essential for the ligand binding and adhesion properties of integrins. It also explains why the recognition sequences of all ligands for integrins contain a carboxyl group and why different integrins can recognize the same ligands (Humphries et al., 2003).

1.4. CONFORMATIONAL CHANGES IN INTEGRINS

1.4.1. Ligand induced conformational changes in the headpiece region of integrins

The 3D structures of liganded and unliganded forms of the collagen receptor integrin α_2I domain are presented in Figure 10. In the absence of ligand the αI domain is in the closed conformation. Ligand binding induces a series of conformational changes leading to the opening of αI domain. The most significant structural changes are the following: a) as a result of the movements of the α1 helix and loop 1 (L1) the MIDAS metal ion comes closer to loop 2 (L2) and helix 3 is relocated; b) the C-terminal α7 helix moves significantly (10 Å) downwards, and c) the movements of the α6 and αC helices increase the length of α6 helix by one turn and the short, one-turn αC helix disappears. Unwinding of the αC helix significantly changes the position of tyrosine 285 (Y285). Highly similar conformational changes are seen in the α_MI domain of leukocyte integrin upon ligand binding (Emsley, 2000) suggesting that the ligand

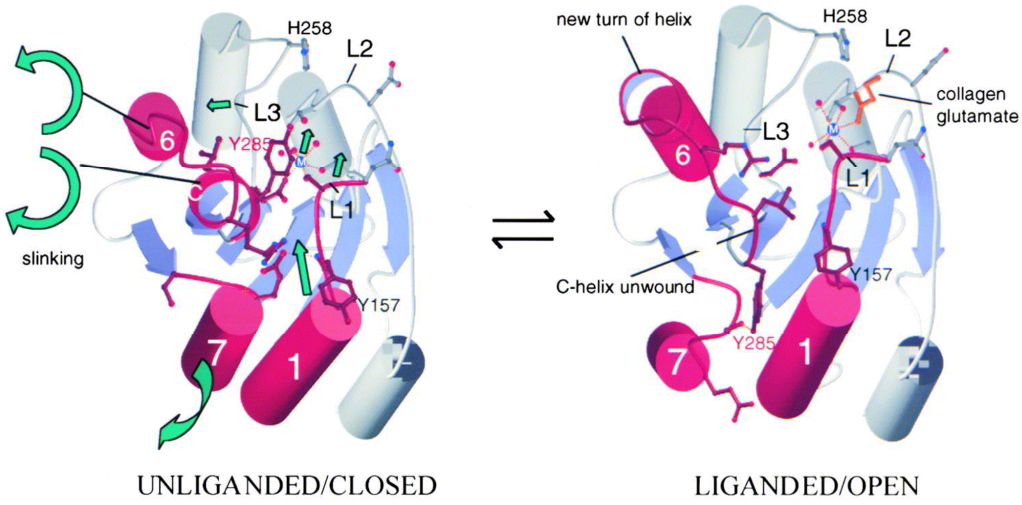

Figure 10. Three-dimensional structure of the collagen receptor α_2I domain with and without ligand. Regions where significant confomational changes occur as a result of ligand binding are shown in red, the MIDAS metal ion is indicated as a blue sphere, and the largest structural transitions are emphasized by green arrows. *(Emsley et al., 2000, Cell 101, 47-56. Reprinted with permission from Elsevier).*

induced structural changes are common and similar in all integrins, which have an αI domain.

Humphries et al. (2003) have listed the following experimental evidence to prove that the open conformation of the αI domain is the active form with high avidity, and that the closed conformation is the less active form with low avidity: a) the mutations stabilizing the open conformation increase the ligand avidity of the αI domain; b) the molecules stabilizing the closed conformation decrease ligand binding and cell adhesion; c) the closed form stabilized by the disulphide bridge is inactive, whereas the open form stabilized by the disulphide bridge is active, and d) the crystal structure of the unliganded αI domain is in the closed conformation, whereas the ligand-bound complex is in the open conformation (Figure 10).

The βI domain of the integrin β subunit and the αI domain of the integrin α subunit belong to the same VWA domain family (see section 1.2.2.) and thus their 3D structures are very similar. Furthermore, macromolecular ligand binding to αI-less integrins induces similar conformational changes in the βI domain as described above for the αI domain, including significant movements of the MIDAS metal ion, specific loops and α1 and α7 helices. The movement of the C-terminal α7 helix of the βI domain causes changes in the contact surface of βI and hybrid domains, swinging out hybrid and PSI domains, which will lead to the separation of the integrin legs and to effects seen all the way down to the cytoplasmic tails of the integrins. In αI-less integrins ligand binding induces structural movements also in the ADMIDAS and LIMBS metal ion binding sites (Figures 6 and 7), which are absent in the αI domains (Xiao et al., 2004). Furthermore, the occupation state of the three metal ion binding sites apparently have effects on the structural changes occurring in the βI domain (Mould et al., 2003; Mould & Humphries, 2004b).

Figure 11A shows a schematic presentation of the drastic conformational changes occurring in integrins lacking an αI domain. Integrins with αI domains are structurally somewhat more complicated than αI-less integrins as they have an extra domain. The three-dimensional ectodomain structure for one integrin including an αI domain, $\alpha_x\beta_2$, has recently been solved (Xie et al., 2010). This structure is unliganded and so it is not yet known what kinds of conformational changes ligand binding to the αI domain exerts on the extracellular region. However, it has been suggested that ligand induced structural changes in αI domains would result in a similar series of conformational changes as have been seen upon ligand binding in the extracellular region of integrins lacking an αI domain (Alonso et al., 2002). Collagen binding to the αI domain leads to numerous structural changes (Figure 10), of which Figure 11B shows only the

significant downward movement of the C-terminal α7 helix. As a result of this movement a highly conserved glutamate (shown by a yellow sphere in the contact region of αI and βI domain in Figure 11B) in the loop between the β-

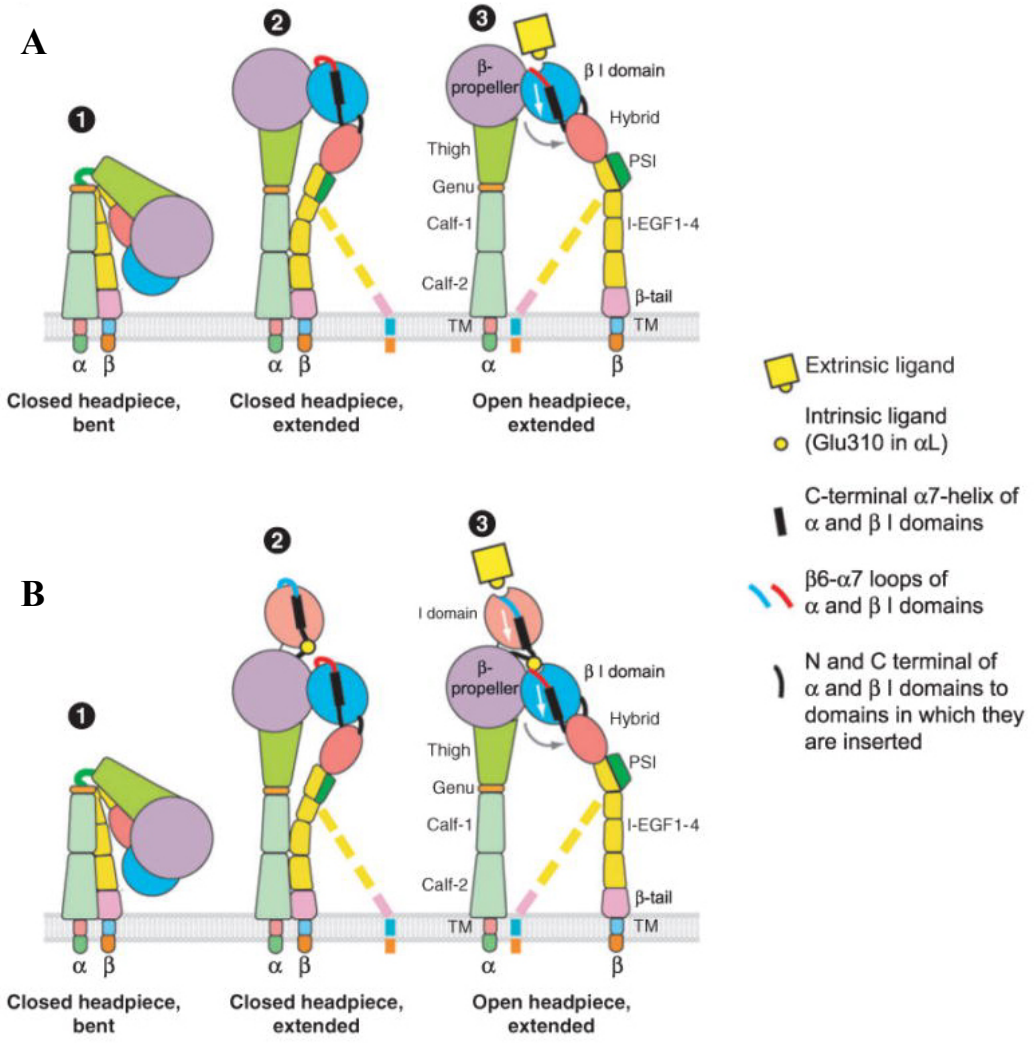

Figure 11. Schematic presentation of the structural effects of ligand binding to integrins. A) Integrins lacking an αI domain. B) Integrins having an αI domain. Ligand induced allosteric swing-out of the hybrid domain leads to leg separation. The lower leg region of the β subunit is highly flexible existing in various conformations (section 1.4.2.) shown by solid and dashed representations. *(Luo et al., 2007, Annu. Rev. Immunol. 25, 619-647. Reprinted with permission from Elsevier).*

propeller and αI domains is relocated so that it binds to the MIDAS metal ion of the βI domain, which in turn results in a similar series of conformational changes in the extracellular region of the β subunit as described below for integrins lacking an αI domain. According to this model in integrins with an αI domain the ligand-activated αI domain has the same kind of effect on the structure of the extracellular region as direct ligand binding has on those integrins lacking an αI domain; i.e., the αI domain acts like an endogenous ligand for the βI domain (Alonso et al., 2002) (Figure 11). Conformational changes occurring in the αI domain would thus be mediated via the βI domain to the structure and function of integrins. However, it has also been shown that the αI domain can be allosterically regulated by the βI domain (Alonso et al., 2002; Yang et al., 2004a; Mould & Humphries, 2004b). Based on the 3D structure of the unliganded $\alpha_x\beta_2$ the model shown in Figure 11B, it is still valid, except that the αI domain is inserted into the β-propeller domain by a flexible linker allowing more flexibility for the αI:βI interaction (Xie et al., 2010) than shown in Figure 11B, where the αI domain is rigidly positioned above the β-propeller domain.

1.4.2. Conformational changes in the extracellular region of integrins

Figure 11 shows the three conformations generally presented for integrins. These structural changes have significant effects on ligand binding properties. The bent conformation is the least adhesive form, whereas the extended conformation with the open headpiece is the most adhesive form. Ligand binding induces significant changes in the positions of the hydrid and PSI domains causing the separation of the integrin leg regions (Figure 11). In this process the contact surfaces between the βI-Hydrid and Hydrid-PSI domains function as flexible knees like the region between the hybrid and EGF3 domains and the link

between the thigh and calf1 domains do when the extended integrin bends to the less adhesive form.

All the solved 3D structures for the integrin extracellular region are in the bent conformation (Figure 12A) irrespective of the presence or absence of ligands (Xiong et. al., 2001, 2002, 2009; Zhu et al., 2008; Xie et al., 2010) indicating that an isolated extracellular region of an integrin is able to bind ligands in the bent conformation. Proof for the existence of the extended forms of integrins (Figure 11) has been obtained by electron microscopy images showing bent and extended conformations with both αI-less integrins and with integrins having an αI domain (Takagi & Springer, 2002; Zhu et al., 2008; Chen et al., 2010, 2012; Xie et al., 2010; Springer & Dustin, 2012). Ligand-induced swing-out of the hydrid and PSI domains (Figure 11) has been observed with $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_X\beta_2$, $\alpha_L\beta_2$ and $\alpha_4\beta_7$ (Xiao et al., 2004; Luo et al., 2007; Zhu et al., 2008; Chen et al., 2010, 2012; Xie et al., 2010; Springer & Dustin, 2012; Nagae et al., 2012; Yu et al., 2012), but the extent of this swing-out seems to vary significantly between different integrin forms (Askari et al., 2009).

Figure 12 shows two models, which differ in the way that the C-terminal end of the extracellular leg region of the integrin, containing the calf1 and calf2 domains of the α subunit and the EGF3-4 and βTD domains of the β subunit (see Figure 4), are located in the bent form with respect to the membrane. In the Switch blade model (Beglova et al., 2002), also known as the Flick-knife model (Liddington, 2002) the feet of the bent integrin are orthogonal to the plasma membrane and the extracellular ligand binding site, the head of the integrin, is very close to the plasma membrane. In the Angle-poise model (Hynes, 2002) the feet are not orthogonal to the plasma membrane and thus the head is more distant from the plasma membrane. In the Angle-poise model the

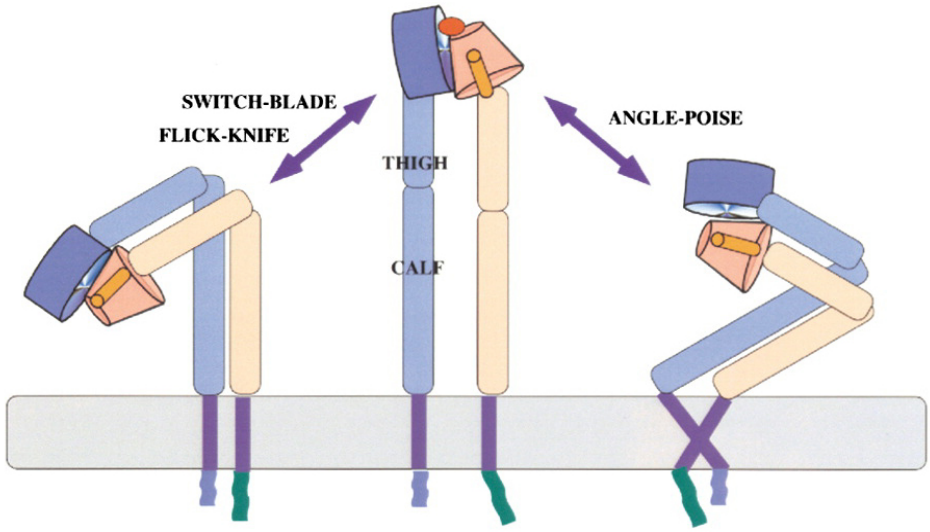

Figure 12. Switch-blade and Angel-poise model presentations of the bent form of integrins. *(Hynes, 2002, Cell 110, 673-687. Reprinted with permission from Elsevier).*

extracellular ligand binding site of the bent integrin form is more accessible, at least for a small molecular ligand, than in the Switch blade model. Even though the Switch blade model is the model mainly presented and preferred in integrin literature, the crystal structures of the extracellular regions of integrins have been determined without the plasma membrane, and so the Angle-poise model cannot be excluded. It is clear from Figure 12 that the details for the bent integrin to stand up into an extended, straight form would be somewhat different in these two models. In both of these models the integrin has to at least partially stand up before the ligand-induced conformational changes in the headpiece region leading to the swing-out of the hybrid domain and leg separation (Figure 11) can happen. A Deadbolt model has been proposed in which a long loop of the βTD domain (Figure 4A) would act as a lock in the bent form preventing the conformational changes in the α7 helix of the βI domain and in the loop preceding that helix, thus also preventing the transition of the bent form into the extended form (Xiong et al., 2003; Arnaout et al..,

2005). In the Deadbolt-model intracellular signals interacting with the cytoplasmic domain of the integrin would open the lock leading to integrin unbending and activation. However, recent structural, functional and mutational studies with $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, and $\alpha_X\beta_2$ have shown that this βTD loop is not important for integrin activation, thus disproving the Deadbolt model (Zhu et al., 2007b; Xie et al., 2010; Shattil et al., 2010).

The flexible knees in the extracellular region allow, in principle, for the integrin to exist in a great number of different conformations with the bent and fully extended, leg-separated conformations being only the two ultimate stages. Figure 13 shows examples of putative conformations. These types of conformations are seen by electron microscopy (Zhu et al., 2008; Chen et al.,

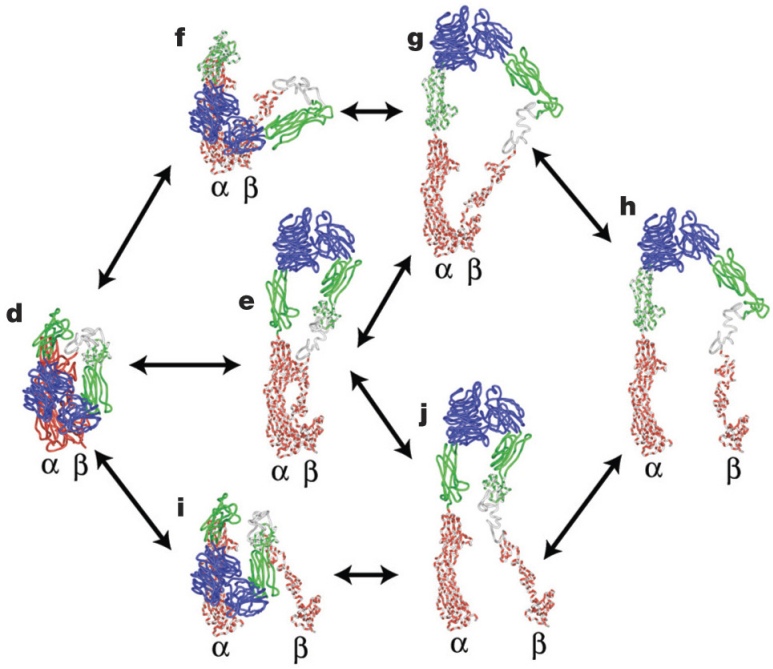

Figure 13. Putative conformations between the bent and extended, leg-separated forms of integrins. *(Xiao et al., 2004, Nature* 432, 59-67. *Reprinted with permission from Macmillan Publishers Ltd.).*

2010, 2012; Xie et al., 2010; Springer & Dustin, 2012). It has been shown that the distance of the extracellular head-piece of $\alpha_4\beta_1$ integrin from the plasma membrane differs depending on how the integrin is activated and that the further the head-piece of the integrin is from the membrane the higher the avidity the integrin has for its ligands (Chigaev et al., 2003, 2007). The βI domain also exists in several different conformations differing from each other, for example, in the degree of the shift that has occurred in the α7 helix position (Figure 11A) and in the ligand binding avidity (Yang et al., 2004b; Luo et al., 2004; Mould & Humphries, 2004b). Furthermore, a pulling force on the integrin extracellular domain may have effects on integrin extension (Alon & Ley, 2008; Kim et al., 2011) and a lateral force created by the binding of the integrin to a growing actin cytoskeleton may have effects on the degree of leg separation (Zhu et al., 2008; Schürpf & Springer, 2011; Springer & Dustin, 2012). Accordingly, it seems that integrins exist in several conformational forms and that the degree of unbending is both agonist- and integrin-specific and correlates with ligand binding affinity (Chigaev et al., 2003, 2007 Mould & Humphries, 2004b; Askari et al., 2009). As described below membrane-embedding of integrins is an essential element for regulating their affinity (Lau et al., 2009; Shattil et al., 2010; Kim et al., 2011), which means that to see physiologically relevant conformations it will be necessary to have intact, membrane-embedded integrins in various states.

1.5. TRANSMEMBRANE AND CYTOPLASMIC DOMAINS OF INTEGRINS

Transmembrane and cytoplasmic domains have a key role in integrin activation and signaling even though they are structurally much simpler than the extracellular region of integrins. In the bent form of integrins the trans-membrane domains of the α and β subunits are in contact with each other (Kim

et al., 2003) (Figures 1 and 11). It has been shown by mutational analysis that disrupting these contacts increases the affinity of the integrin to its ligand, and vice versa strengthening the contacts decreases the affinity (Hughes et al., 1996; Hynes, 2002; Kim et al., 2003; Humphries et al., 2003; Travis et al., 2003; Li et al., 2005; Zhu et al., 2007c). The 3D structure for the transmembrane domain (TMD) of $\alpha_{II}\beta_3$ embedded in a lipid bilayer has been solved by NMR (Lau et al., 2009)(Figure 14). The TMD includes one α helix from both subunits. The 24-residue-long transmembrane helix of the α_{II} subunit is perpendicular to the lipid bilayer, whereas the 29-residue-long β_3 transmembrane helix is tilted. The 25° crossing angle between the α and β transmembrane helices facilitates the

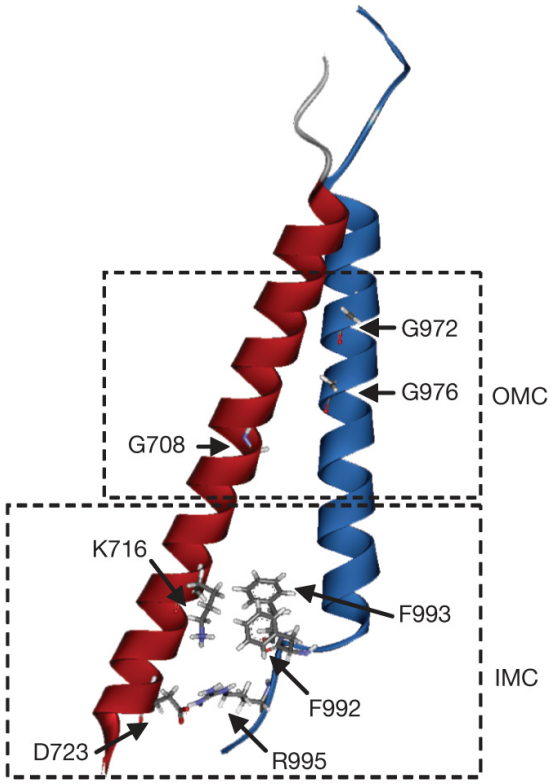

Figure 14. NMR-structure of $\alpha_{II}\beta_3$ transmembrane domain embedded in a lipid bilayer. OMC and IMC refer to the outer and inner membrane clasp, respectively, briefly described in the text. The transmembrane helices of the α_{IIb} and β_3 subunit are shown in blue and red, respectively. This 3D structure was solved by Lau et al. (2009). *(Kim et al., 2012, Nature 481, 209–213. Reprinted with permission from Macmillan Publishers Ltd.).*

specific interactions to occur simultaneously close to the outer membrane and the inner membrane side of the lipid bilayer. The outer membrane clasp (OMC) includes packing of glycines (G972 and G976 of α_{II} and G708 of β_3) and the inner membrane clasp (IMC) includes packing of two phenylalanines (F992 and F993) of α_{II} ,which mediates interhelical packing and an electrostatic interaction between arginine 995 of α_{II} and aspartate 723 of β_3 (Figure 14). When the crossing angle is changed, for example, by proteins binding to the cytoplasmic tails of integrins (see section 1.6.) both OMC and IMC interactions cannot occur at the same time, which leads to the dissociation of the two transmembrane helices (Lau et al., 2009; Shattil et al., 2010; Kim et al., 2011). Interestingly, within the lipid bilayer in the IMC region there is one conserved lysine in the β subunit (K716 in β_3). A positively charged residue in a hydrophobic lipid environment is energetically unfavourable. To minimize this thermodynamic problem Lys716 is extended and the charged ε-amino group is close to the negatively charged phospholipid head groups. This kind of behaviour of lysine and arginine is quite common in transmembrane domains and is called snorkeling. It was recently shown by NMR spectroscopy and mutational analysis that the snorkeling Lys716 of $\alpha_{II}\beta_3$ is an important residue for the 25% crossing angle (Figure 14) and thus it was also suggested that snorkeling regulates integrin transmembrane signaling (Kim et al., 2012).

With the exception of β_4 the cytoplasmic domains of the integrin α and β subunits are quite short (about 15-50 residues; see page 19). The amino acid sequences of the cytoplasmic domains of the β subunits are better conserved than those of the α subunits. This supports the idea that the cytoskeleton and the components of intracellular signaling pathways interact with the C-terminal domain of the β subunit, whereas the cytoplasmic domain of the α subunit has a regulatory role (Humphries et al., 2003). Hovever, collagen receptor integrins

($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$), for example, share the same β subunit and so differences in their interactions with the intracellular signaling apparatus are due to different α subunits (White et al., 2004). Most proteins found to interact with the cytoplasmic tails of integrins bind to the β subunit and quite little is known about proteins interacting with the cytoplasmic tails of integrin α subunits (Barzyk et al, 2010; Shattil et al., 2011; Margadant et al., 2011; Rantala et al., 2011; Pellinen et al., 2012).

The only conserved sequence in the cytoplasmic domain of human integrin α subunit is a membrane proximal GFFKR (Gahmberg et al., 2009). This sequence is important for keeping the transmembrane domains of the α and β subunits together in the bent, low affinity form (Figure 1). The conserved arginine of this sequence belongs to the IMC forming an electrostatic interaction with a specific aspartate in the β subunit (R995 of α_{II} and D723 of β_3 in Figure 14). The β subunits contain two conserved sequences, the membrane proximal (MP) NPxY and the membrane distal (MD) NxxY, which are binding sites for specific proteins, such as talins and kindlins, respectively, regulating integrin activity (see below)(Moser et al., 2009; Shattil et al., 2011; Margadant et al., 2011).

Despite the fact that the cytoplasmic domains of integrins are much smaller/shorter than the extracellular region, the 3D structure of the cytoplasmic region is not known well. The 3D structure of the cytoplasmic region of $\alpha_{IIb}\beta_3$ integrin determined by NMR indicates that the regions adjacent to the transmembrane domains are in the α-helical structure in both subunits, whereas the C-terminal ends are unstructured (Vinogradova et al., 2002). This suggests that the C-termini of both subunits are flexible forming alternative 3D structures depending on the macromolecules they are interacting with.

1.6. INTEGRIN ACTIVATION AND REGULATION

Although integrins are signaling adhesion receptors, they do not have enzyme activities in their cytoplasmic domains, as do many other signaling receptors. The conformational changes mediated across the membrane act as bidirectional signals in integrins. Integrins interact with several cytoplasmic proteins (Hughes & Pfaff, 1998; Hemler, 1998; Liu et al., 2000; van der Flier & Sonnenberg, 2001; Miranti & Brugge, 2002; Brakebusch & Fässler, 2003; Margadant et al., 2011; Shattil et al., 2010; Kim et al., 2011; Serrels et al., 2012; Lawson et al., 2012; Pellinen et al., 2012). These interactions are modulated by conformational changes occurring in integrins and thus structural changes are mediated both into the extracellular and intracellular space via a sophisticated network of interactions (Giancotti & Ruoslahti, 1999; Calderwood et al., 2000; Schwartz & Ginsberg, 2002; Miranti & Brugge, 2002; Legate et al., 2006; Zhang & Wang, 2012). Talin is a cytoplasmic protein with a key role in integrin activation and in mediating conformational signaling of integrins to the cytoskeleton. Vertebrates have two talin isoforms (talin 1 and 2), whereas lower eukaryotes have one talin corresponding to talin 1 (Senetar et al., 2007). Talins are antiparallel, homodimeric proteins with a subunit size of 270 kDa (Calderwood & Ginsberg, 2003). They are composed of an N-terminal head (residues 1-400), a linker region (residues 401-481) and a long C-terminal rod domain (residues 482-2541). The talin head interacts with the cytoplasmic tail of the integrin β subunit and the rod interacts with the actin microfilament of the cytoskeleton (Calderwood et al., 2003; Calderwood & Ginsberg, 2003; Travis et al., 2003; Gingras et al., 2009). The head contains a FERM (4.1, ezrin, radixin, moesin) domain including three subdomains (F1-F3) and an F0 domain (Gingras et al., 2009; Anthis et al., 2009). The F3 subdomain has a so-called phosphotyrosine-binding fold (PTB) by which talin first binds to the membrane proximal conserved NPxY sequence and then the F3 binds to the membrane

proximal helical region of the β subunit breaking down the IMC contacts shown in Figure 14. Furthermore, four basic highly conserved, positively charged residues on the surface of the head F2 subdomain interact with the membrane re-orienting talin, which results in a 20° change in the crossing angle between the α and β transmembrane helices leading to the dissociation of the transmembrane domains and the activation of the integrin (Anthis et al., 2009) (Figure 15). This novel combined effect of the F3 and F2 subdomains makes talin unique among the many other PTB-domain containing proteins of the cytoskeleton and signaling pathways and it also makes sure that no other PTB-domain containing protein will start the integrin activation process. Talin binds at least to β_1, β_2, β_3, β_5 and β_7 integrins (Margadant et al., 2011); i.e., 21 of the 24 mammalian integrins (Figure 2) and it is thus a general activator protein of most, if not all, integrins.

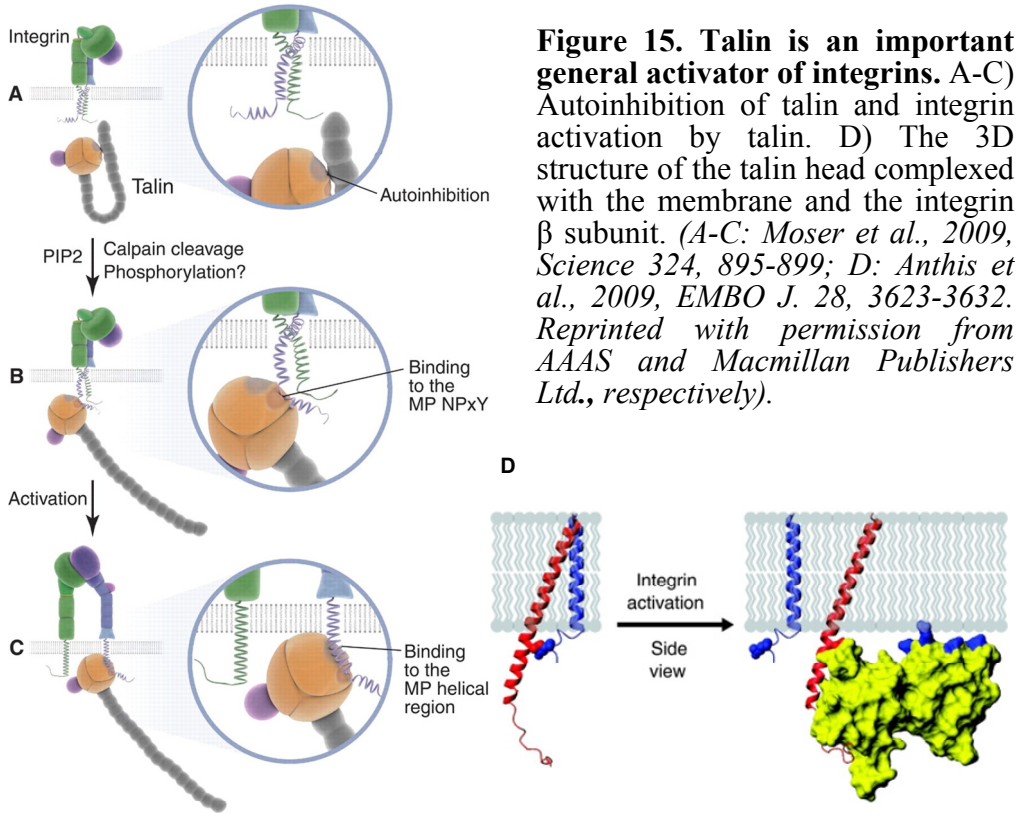

Figure 15. Talin is an important general activator of integrins. A-C) Autoinhibition of talin and integrin activation by talin. D) The 3D structure of the talin head complexed with the membrane and the integrin β subunit. *(A-C: Moser et al., 2009, Science 324, 895-899; D: Anthis et al., 2009, EMBO J. 28, 3623-3632. Reprinted with permission from AAAS and Macmillan Publishers Ltd., respectively).*

Talin is strictly controlled by an autoinhibition mechanism to ensure that integrin activation happens only under specific physiological conditions. Talin exists in two forms differing in their activities. In the inactive form the PTB domain responsible for binding to the integrin is buried in a three dimensional structure so that it is unable to bind to the integrin (Wegener et al., 2007; Goksoy et al., 2008). Activation of talin by inositol phospholipids and/or by proteolytic activation results in conformational changes making the PTB domain accessible for the integrin. Proteolytic activation of talin is catalyzed by calpain (Paolo et al., 2002; Travis et al., 2003) (Figure 15). Calpains are Ca^{2+}-ion dependent proteases, which have a cysteine in their active site, and which exert various effects in cells (Zatz & Starling, 2005). Activation of talin by inositol phospholipids leads to integrin activation, whereas proteolytic activation in which the talin head is cleaved from the rod also leads to the loss of the integrin connection with the actin microfilament of cytoskeleton and focal adhesion disassembly (Legate et al., 2009; Moser et al, 2009). At least with β_1 and β_3 integrins phosphorylation of the tyrosyl residue of the conserved NPxY motif has been shown to inhibit talin interactions with the cytoplasmic domain of the integrin β subunit (Moser et al., 2009) (Figure 15).

In addition to talins, kindlins are also general and important activators of integrins, and they cooperate with talins (Moser et al., 2009; Shattil et al., 2010; Kim et al., 2011). Mammals have three kindlin isoforms, kindlin-1, -2 and -3 (Meves et al., 2009; Karaköse et al., 2010). Kindlins belong to the group of FERM-domain containing proteins. Kindlins are significantly smaller proteins than talins. They resemble the talin head region except that kindlins possess a pleckstrin homology (PH) domain inserted into the F2 subdomain (a unique feature among FERM domain-containing proteins)(Karaköse et al., 2010). Kindlins bind to the cytoplasmic membrane distal NxxY sequence motif of the

integrin β_1, β_2 and β_3 subunits (Meves et al., 2009; Moser et al., 2009); i.e., 18 of the 24 mammalian integrins. The details for how kindlin binding to the integrin cytoplasmic β-tails helps talin to perform its task in integrin inside-out activation are not known. Furthermore, kindlins are also required for outside-in activation, in which they together with other proteins, such as integrin-linked kinase (ILK), migfilin, focal adhesion kinase (FAK) and α-actinin, indirectly link integrins to the actin cytoskeleton and are thus involved in integrin-induced signaling (Meves et al., 2009; Moser et al., 2009; Böttcher et al., 2009). Kindlins seem to have differential effects on specific integrin heterodimers (Harburger et al., 2009; Manevich-Mendelson et al., 2009; Karaköse et al., 2010).

In addition to talins and kindlins numerous other intracellular proteins are involved in the regulation of integrin inside-out activation by having either activating/stimulatory [ILK, migfilin, Rap1, Rap1-GTP-interacting adaptor protein (RIAM), for example] or inhibitory [Dok-1, Filamin, Calcium and integrin-binding protein 1 (CIB1), integrin cytoplasmic domain-associated protein 1 (ICAP-1), SHARPIN] effects on this process (Wegener & Campbell, 2008; Böttcher et al., 2009; Gahmberg et al., 2009; Shattil et al., 2010; Kim et al., 2011; Rantala et al., 2011; Zhang & Wang, 2012). Pellinen et al. (2012) just recently used a functional screen to identify 13 activators and 10 inhibitors of β_1 integrin regulation.

Integrin inside-out activation is a complex and tightly regulated process, which is terminated by the talin-induced dissociation of integrin transmebrane domains (Kim et al. 2011; Shattil et al. 2010; Kahner et al., 2012)(Figure 14). Dissociation of the transmembrane domains initiates a series of conformational changes from the cytoplasmic domains to the extracellular region (inside-out

signaling) leading to a switch from the closed conformation to the open conformation, which makes it possible for the integrin to bind to the ECM ligand. The binding of the integrin with the ECM ligand induces another set of conformational changes in the integrin (outside-in signaling)(Figure 11), which results in linking the integrin cytoplasmic domains to the actin microfilament of the cytoskeleton (Calderwood & Ginsberg, 2003; Travis et al., 2003; Moser et al., 2009; Böttcher et al., 2009). This in turn induces the clustering phenomenon, in which several integrins will be clustered close to each other on the cell surface. The clustering increases the adhesion of integrins to their ECM ligands (Hato et al., 1998; Carman & Springer, 2003). As a result of this clustering the integrins will also be tightly connected to a highly complicated network of cytoskeletal proteins, in which intracellular signaling molecules will join leading to the formation of a huge focal adhesion complex (van der Flier & Sonnenberg, 2001; Zamir & Geiger, 2001; Ling et al., 2002; Geiger et al., 2009; Serrels & Frame, 2012; Lawson et al., 2012). The formation of the focal adhesion complex will have effects on various intracellular signaling pathways, which in turn has many kinds of effects on the cellular functions, such as proliferation, survival and gene expression, for example (Giagnotti & Ruoslahti, 1999; Calderwood et al., 2000; Schwartz & Ginsberg, 2002; Miranti & Brugge, 2002; Legate et al., 2006, 2009; Wehrle-Haller, 2012). Furthermore, integrin trafficking mechanisms are important in regulating adhesion (dis)assembly and migration in adherent cells (Margadant et al., 2011).

Even though integrins are regulated by bidirectional conformational changes, clustering and trafficking, the role of each of these events and regulatory mechanisms involved differ significantly between different integrins and cell types (Margadant et al., 2011; Kim et al, 2011). Furthermore, integrins cooperate in many ways with other receptors, including growth factor receptors. So to undertand the overall processes involving integrins and their

consequences one has to take into account all these versatile interactions and regulatory mechanisms (Ivaska & Heino, 2011).

2. AIMS OF THE STUDY

The main topic for this Ph.D. thesis was to study the effects of the activation of the collagen receptor integrin on its ligand binding properties. Collagen binding by the leukocyte specific integrins was also studied. These studies will help in understanding the ligand selectivity of integrins and the partially overlapping roles of the collagen receptor and leukocyte specific integrins, as well as in developing more specific drugs targeted to collagen receptor integrins. Small molecule drugs targeted to these integrins could be used to treat cancers, inflammations and diseases of the blood circulatory system. The four specific aims of this Ph.D. thesis work were the following:

Aim 1: To structurally and functionally characterize the activated form of the collagen receptor α_1I domain.

Aim 2: To find out the effects of activation on the ligand binding and selectivity of collagen receptor integrins.

Aim 3: To find out the effects of activation on the ligand binding and selectivity of leukocyte specific integrins.

Aim 4: To develop small molecule inhibitors for collagen receptor integrins.

3. MATERIALS AND METHODS

3.1. MATERIALS

The following ligands and inhibitors were used. The collagen subtypes were purchased from the following companies: Human collagens I, II, III, IV, V and VI from Biomarket (Turku, Finland), rat collagen I from Sigma Aldrich (Helsinki, Finland) and mouse collagen IV from Becton Dickinson (Helsinki, Finland). Recombinant human collagen IX was a kind gift from Drs. J. Jäälinoja and L. Ala-Kokko (University of Oulu, Finland). Mouse laminin-111 was purchased from Sigma Aldrich (Helsinki, Finland), human laminin-211 was from Chemicon International, AH Diagnostics (Helsinki, Finland). Human recombinant laminins -411 and -511 were produced as described earlier by Kortesmaa et al. (2002) and Doi et al. (2002), respectively. Human intercellular adhesion molecule-1 (ICAM-1) and inactivated complement fragment 3 (iC3b) were obtained from R&D Systems (Minneapolis, MN, USA) and Calbiochem/Merck (Nottingham, United Kingdom), respectively. The GFOGER peptide was synthetized by Auspep (Melbourne, Australia). Tetracycline compounds described in paper III of this thesis (Käpylä et al., 2007) were kindly produced and purified by Kaisa Palmu (University of Turku) from the *Streptomyces* strains purchased from Lividans Ltd. (Turku, Finland).

Other materials used are described in the four original articles (Papers I-IV).

3.2. METHODS

3.2.1. Cloning and mutagenesis of the human integrin α subunits and αI domains

The cDNAs of the collagen receptor integrin α_1I domain including amino acids ^{138}ECS…LEATA^{338} (Nykvist et al., 2000) and the α_2I domain including residues ^{125}PDGF…EGTV^{339} (Ivaska et al., 1999) had been previously cloned into the pGEX-4T and pGEX-2T vectors (Amersham Biosciences, Uppsala,

Sweden), respectively. In this work the αI domains of leukocyte specific integrins were cloned into the pGEX-4T vector (paper IV: Lahti et al., 2012).

The full-length human α_1 integrin constructed in the pcDNA3.1/Z-2 plasmid (Invitrogen, Carlsbad, CA, USA) was generously provided by Dr. Pauli Ollikka (Biotie Therapies Corp., Turku, Finland). The point mutations to both full-length α subunits (α_1 and α_2) and αI domains were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All mutations were verified by DNA sequencing. The plasmid constructs were purified with the Qiagen Maxi kit (Qiagen Nordic, Helsinki, Finland) and transfected into Chinese hamster ovary (CHO) cells (American Type Culture Collection, ATCC, Rockville, MD, USA) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). The CHO cell clones expressing human α_1 or α_2 integrin were created as described previously (Nykvist et al., 2000).

3.2.2. Cell adhesion assays

In paper I the attachment and spreading of $\alpha_1\beta_1$ (human α_1, hamster β_1) expressing CHO cells on collagen I and IV matrices were tested with the xCELLigence real-time cell analyzer (RTCA; Roche Diagnostics GmbH, Penzberg, Germany). This technology measures impedance at the bottom of a microtiter plate well and allows estimation of the progression of cell attachment and spreading. The same technique was used in paper IV to study the adhesion and spreading of leukocytes and human promyelocytic leukemia (HL-60) cells (ATCC, Rockville, MD, USA). In paper III adhesion of CHO cell clones expressing α_2 integrin on a collagen I matrix was measured by physically separating the adherent and nonadherent cells followed by the detection of living adherent cells using the Oncogene cell viability kit.

3.2.3. Protein expression and purification

αI domains were expressed in *Escherichia coli BL21 Tuner*™ (Novagen Inc., Madison, WI, USA) and purified as gluatiothione-S-transferase (GST) fusions as described in paper II (Tulla et al., 2008) for α_1I and α_2I domains. For the production of proteins for crystallizations gel filtration was used after affinity chromatography (Paper I). *E. coli* cells were grown both in bottles in a shaker at 37°C (Papers I-IV) and in a Bioengineering fermentor (Bioengineering AG, Wald, Switzerland) (Paper I). The purity of the protein was checked by electrophoresis on 8–25% gradient polyacrylamide gels in the presence of 0.55% sodium dodecyl sulphate using the Phast System (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein concentrations were measured by the Bradford (1976) method based Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

3.2.4. Solid phase binding assays

Ligand binding measurements were carried out with a time-resolved fluorescence immunoassay (TRFIA) in 96-well plates as described in papers I-IV. In the assay a Delfia® Europium labeled anti-GST antibody (Wallac PerkinElmer, Turku, Finland) was used and the signal was detected by a time-resolved fluorescence spectrophotometer (Victor3 multilabel counter, Wallac PerkinElmer, Turku, Finland). In the binding assays different types of collagen, short, synthetic triple helical GFOGER peptide (Knight et al., 2000; Käpylä et al., 2004), as well as non-collagenous compounds, such as laminins, intercellular adhesion molecule-1 (ICAM-1) and inactivated complement fragment 3 (iC3b) were used as ligands.

3.2.5. Crystallization and data collection

Crystallization was achieved with the hanging drop vapor diffusion method under the conditions described in paper I. The crystal diffracted to 1.9 Å, and the data were collected on a ADSC Quantum Q210 detector installed on beamline ID 14-1 at the European Synchrotron Radiation Facility (ESRF, France). Crystallization and data collection were carried out in Dr. Tiina Salminen's group by Eva Blight and Anna-Maria Brandt.

3.2.6. Structure determination, model building and refinement

The 3D structure of the active variant of the α_1I domain (E317A/C139S) without ligand was solved as described in paper I. The homology model of the E317A variant was built in paper II using the coordinates of the wild type α_1I domain (Nymalm et al., 2004) and the ligand-bound integrin α_2I domain (Emsley et al., 2000) as templates. X-ray analysis and homology modeling were carried out by the research groups of Drs. Tiina Salminen and Mark S. Johnson, respectively.

3.2.7. Docking of small molecule inhibitors into the closed conformation of the α_2I domain

To search for small molecular inhibitors of collagen receptor integrins putative inhibitors were docked into the closed conformation of the α_2I domain by the program FlexX in Sibyl 6.9 (Tripos, St. Louis, MO, USA). This task was performed in Professor Mark S. Johnson's group.

4. RESULTS AND DISCUSSION

As described in section 1.2. integrins are very long, integrally membrane-bound, transmembrane proteins and are thus very hard to express and purify in intact form. Fortunately, the αI domain, which is present in the α subunit of the extracellular head region of collagen-binding and leukocyte specific integrins (section 1.2.2.) and is responsible for ligand binding, folds independently into its native, functional conformation. Accordingly, the structural and ligand binding properties of these two integrin groups can be reliably studied by soluble αI domains (Humphries, 2000; Hynes, 2002). Like intact integrins, the soluble αI domains also exist in a closed, low affinity form and an open, high affinity form (section 1.4.1.), and so it is possible to use the isolated αI domain in order to understand the factors and mechanisms of the activation/deactivation of these two groups of integrins. The integrin αI domain can be activated by ligand binding (Emsley et al., 2000) or by various kinds of site-specific mutations which stabilize the open, activated conformation (Li et al., 1998; Xiong et al., 2000; Shimaoka et al., 2002; Aquilina et al., 2002; Vorup-Jensen et al., 2003; Shimaoka et al., 2003; McCleverty & Liddington, 2003; Siljander et al., 2004).

The purpose of this thesis was to study the structure and function of αI domains in collagen receptor and leukocyte specific integrins with special emphasis on the effects of integrin activation on ligand binding and selectivity. The results are briefly summarized in sections 4-5. For more details, please, see the original publications in section 8.

4.1. STRUCTURE DETERMINATION OF THE ACTIVATED, LIGAND-FREE α_1I DOMAIN SUGGESTED A THIRD CONFORMATIONAL STATE OF THE αI DOMAIN (Paper I: Lahti et al, 2011)

In paper II of this Ph.D. thesis the gain-of-function mutation of the α_1I domain (E317A) was shown to increase collagen avidity (Tulla et al, 2008; see section 4.2.). By testing the effects of integrin activation in cell lines overexpressing human $\alpha_1\beta_1$ and its activated mutants we showed in paper I that conformational activation of integrin $\alpha_1\beta_1$ leads to high-avidity cell adhesion to collagens I and IV in human integrin $\alpha_1\beta_1$ transfected CHO-cell lines (Figure 1 in Lahti et al. 2011). Previously the 3D structure of the collagen-binding integrin α_1I domain was solved in a closed, low-affinity conformation (Nymalm et al., 2004). In paper I of this thesis a high-affinity variant (E317A/C139S) of the α_1I domain was constructed, its collagen binding properties were studied and the 3D structure was solved by X-ray analysis at an 1,9 Å resolution (Lahti et al. 2011). The C139S mutation was made to significantly increase protein yield, purity and solubility facilitating crystallizations and X-ray analysis. The mutation did not affect the activation of the αI domain. The corresponding cysteine to serine mutation had been previously made for the α_MI domain of the leukocyte integrin to decrease its aggregation (Xiong et al., 2000). The activating mutation (E317A), which breaks the salt bridge E317:R287 caused drastic conformational changes resulting in the unwinding of the αC helix into a loop structure and large movements of residues R287 and Y285 (Figure 4 in Lahti et al. 2011). In the closed conformation of α_1I domain Y285 covers the metal ion-dependent adhesion site (MIDAS), from which it has moved aside in this high-affinity conformation making MIDAS more accessible for the ligand (Figure 4B in Lahti et al. 2011). This is the first 3D structure for the activated α_1I domain and more generally the first 3D structure for an activated collagen

receptor αI domain without ligand. The 3D structure of the high-affinity variant α_1I E317A was previously predicted by molecular modeling (paper II: Figure 2 in Tulla et al, 2008) using the coordinates of the wild type α_1I domain (Nymalm et al., 2004) and the ligand-bound integrin α_2I domain as templates (Emsley et al., 2000). In the 3D structure of the ligand-free α_1I C139S/E317A solved in paper I the conformational changes compared to the closed form were smaller and somewhat different from those expected based on the model of the activated form described in paper II (Tulla et al., 2008) and seen for the α_2I domain when the closed and activated, ligand-bound forms were compared (section 1.4.1.; Emsley et al., 2000). The E317A mutation results in the unwinding of the αC helix as seen with the activation of α_2I, but this mutation moved the MIDAS metal ion toward loop 1, instead of loop 2 as seen in the activation of α_2I. The α7 helix, which has significantly moved downward in the open α_2I structure, has not changed its position in the activated α_1I variant (Figure 5 in Lahti et al., 2011). Interestingly, compared with the hexacoordination of the MIDAS metal in previously published α_1I and α_2I structures, the metal ion in our activated α_1I structure is pentacoordinated (Figure 3 in Lahti et al., 2011). Accordingly, our results suggest that the α_1I domain has at least three main conformations: closed, intermediate and open. The structure of the activated α_1I variant solved in this work is clearly different from the intermediate form of the α_LI domain (Shimaoka et al., 2003). In the activated α_1I domain, all of the structural changes had taken place close to the MIDAS site, whereas in the intermediate-affinity form of the α_LI domain the α7 helix was partially shifted down, and the MIDAS was still closed. Just recently, based on our activated α_1I structure in conjuction with their observations on the dynamic structural changes upon collagen and metal ion binding to the integrin α_1I domain, Weinreb et al. (2012) proposed a model, in which the pentacoordinated form is an important intermediate in the activation of the

integrin αI domain. Mn^{2+} adopts the pentacoordinated conformation more easily than Mg^{2+}, whereas Ca^{2+} is unable to adopt that conformation. This gives structural explanations to why the nonpysiological Mn^{2+} activates integrin more strongly than does the physiological Mg^{2+}, and why Ca^{2+} is incapable of supporting ligand binding.

As described in section 1.4.1 (Figure 11) during integrin activation, a highly conserved glutamate (Glu^{335} in α_1I) of the αI domain acts like an endogenous ligand for the βI domain binding to the metal ion at the MIDAS site of the β_1 subunit. Interestingly, in our cell adhesion assays E317A could activate collagen binding even after mutating the conserved Glu^{335} (Figure 6 in Lahti et al., 2011). This suggests that the stabilization of the α7 helix into its downward position is not required for integrin activation if the α_1I MIDAS site is already open. The activated α_1I domain represents a novel conformation for the αI domain, possibly mimicking the structural state where the E317:R287 ion pair has just broken during the activation of the integrin.

4.2. INTEGRIN ACTIVATION DECREASES THE LIGAND SELECTIVITY OF α_1 AND α_2 COLLAGEN RECEPTOR INTEGRINS (Paper II: Tulla et al., 2008)

4.2.1. Binding to collagen subtypes

Previous studies in Prof. Jyrki Heino's research group have shown that the four collagen-binding integrins, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, have different specificities towards different collagen subtypes (Nykvist et al., 2000; Tulla et al., 2001; Zhang et al., 2003; Käpylä et al., 2004)(section 1.1.2). However, the effect of integrin activation on the selective binding of natural collagen subtypes has not been systematically studied. The activating mutation of the α_2I domain (E318W) breaking the E318:R288 salt bridge (Aquilina et al., 2002),

corresponding to the E317:R287 salt bridge of α_1I described in section 4.1., has been shown to decrease ligand selectivity with different variants of the GFOGER peptide (Siljander et al., 2004)(section 1.3.2.). In Paper II of this thesis we studied the binding of various natural collagen subtypes and laminins (see section 1.1.2.) to α_1I and α_2I wild type (closed conformations) and their corresponding activated mutants (open conformations), E317A and E318W, respectively. We found that the selectivity in ligand recognition is greatly reduced upon activation (Figures 3 and 4 in Tulla et al., 2008). The E317W mutant of the α_1I domain existed in various multimeric forms in solution decreasing the reproducibility of the results. Accordingly, the E317A activation mutation was made for the α_1I domain. This mutant protein did not produce multimers in solution and behaved reproducible in the binding assay described in paper II.

When the closed and open conformations of α_1I and α_2I domains were tested with collagens representing fibrillar collagen (I or II), network-forming collagen (IV), collagen forming beaded filaments (VI) or FACIT collagen (recombinant collagen IX), it was noticed that in every case the binding was better with the open conformations, and for all collagen subtypes except collagen VI the binding levels were about the same in the open conformations, indicating that the selectivity seen with the wild type α_1I and α_2I domains was lost due to the activating mutations (Figures 3 an 4 in Tulla et al., 2008). Thus our results show that conformational activation of both α_1I and α_2I domain leads to high-avidity binding to otherwise disfavoured collagen subtypes. Aquilina et al. (2002) has previously shown that the E318W mutation enhances α_2I binding to collagen I and IV. Deletion of the short αC helix (284-GYLNR-288) in α_2I gave similar results as did α_2I E318W (Lahti, M., unpublished results).

4.2.2. Binding to laminin isoforms

Most integrins are not strictly ligand specific, but instead they are able to bind different kinds of ECM ligands and vice versa many ECM ligands are able to bind to different integrins (Hynes, 2004; Meves et al., 2009). Accordingly, even though there are laminin-binding integrins ($\alpha_3\beta_1$ $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_4$; Figure 2 in section 1.1.1) collagen receptor integrins are also able to bind laminins (Languino et al, 1989; Elices & Hemler, 1989; Ignatius et al., 1990; Wong et al., 1996; Tulla et al., 2001). Laminins (see section 1.1.2) were recently renamed based on the presence of different α, β and γ chains in the trimeric laminin molecule (Aumailley et al., 2005). In paper II the effect of integrin activation was studied with laminins -111, -211, -411 and -511. Laminin-111 (previously known as laminin-1) is the major laminin form expressed during early embryogenesis (Dziadek & Timpl, 1985), laminin-211 (also known as merosin or laminin-2) is expressed in skeletal muscle and in peripheral nerves (Leivo & Engvall, 1988), laminin-411 (previously laminin-8) is a component of vascular basement membranes, especially abundant in the heart (Iivanainen et al., 1997) and laminin-511 (previously laminin-10) is the most widespread laminin isoform and is expressed in epidermis and dermis, for example (Määttä et al., 2001; Pouliot et al., 2002).

In general, the closed conformation of α_1I recognized the tested laminins significantly better than did α_2I. This agrees with the results of Kern et al. (1993) showing that $\alpha_1\beta_1$ binds laminin-111 better than does $\alpha_2\beta_1$. The closed conformation of α_1I binds best to laminins -111, -511 and -211, and weakly, if at all, to laminin-411. Laminins -111 and -511 showed surprisingly tight binding to the wild type α_1I, which was somewhat weaker but comparable with collagen I and IV binding (Figure 1 A-B in Tulla et al., 2008). The wild type α_2I

recognized three laminins poorly in the following order: -111, -511 and -211, but did not recognize laminin-411 (Figure 1C in Tulla et al., 2008).

Tulla et al. (2001) have shown that the R218D and D219R mutations in α_1I and α_2I, respectively, significantly weaken the binding of the αI domain to the collagen subtype preferred by the corresponding wild type domain. In the case of the α_1I domain the R218D mutation (mimicking the α_2I ligand binding surface) abolished the binding of laminins -111, -211 and -511, and drastically decreased collagen IV binding, but had only a small effect on collagen I showing that Arg218 has a key role in ligand binding and selectivity. A mutation in the corresponding, oppositely charged, amino acid in α_2I (D219R mimicking α_1I binding surface) behaved quite differently as it did not have a significant effect on laminin binding. D219R binding to collagen IV was unaltered and binding to collagen I was only slightly decreased compared to wild type α_2I (Figure 1 in Tulla et al., 2008). The effects of these mutations on collagen binding were similar to those presented in Tulla et al. (2001).

Like to the collagen subtypes the α_1I activating mutation (E317A) increased binding to laminins -111, -211 and -511. Laminin-411 binding, which was only slightly tighter than that of the background control bovine serum albumin (BSA), was not effected by the mutations (Figure 6 in Tulla et al., 2008). The dissociation constants for laminin-111 and -211 binding to the α_1I E317A mutant were about the same (~70 nM and ~78 nM, respectively), whereas the wild type α_1I domain bound laminin-111 weakly (K_d ~ 556 nM), but did not bind laminin-211. This indicates that the α_1I E317A activating mutation not only increased laminin binding but also reduced the selectivity between different laminin isoforms. Interestingly, the activating E318W mutation drastically increased binding of laminins -111, -211 and -511 to the α_2I domain,

which only poorly recognized laminins in the wild type, closed conformation (Figure 5 in Tulla et al., 2008). These results clearly showed that activation is required for the α_2I domain to significantly bind laminins -111, -211, and -511. An earlier report has also suggested that binding of the α_2I domain to laminin-111 is enhanced after activation (Aquilina et al., 2002). Laminin-411 binding to the α_2I domain remained insignificant also with the activated mutant. The lack of laminin-411 binding to both the closed and open conformations of the α_1I and α_2I domains may be due to the truncated N-terminus of the α4 chain in laminin-411.

The αC-helix in the αI domain is unique for collagen receptor integrins. It has been shown to unwind and move away from the proximity of the metal binding site either due to ligand binding to α_2I (Emsley et al., 2000)(see section 1.4.1) or as a result of the activating mutation of α_1I (Lahti et al., 2011; section 4.1.). The exact role of the αC-helix is uncertain but it has been suggested to participate in ligand recognition or in the regulation of integrin activation (Käpylä et al., 2000; Tulla et al., 2008). Based on our results we proposed that the αC-helix may act in the "preselection" for ligands before αI domain activation and that a tyrosine residue (Y285 in α_1I and α_2I) of the αC-helix has an important role in this process. If the αI domain is activated before ligand binding no preselection can occur, since the αC helix has disappeared and the metal ion and residues involved in ligand recognition are already exposed for immediate binding. Accordingly, a broader selection of motifs is accepted, which is seen by lower selectivity in ligand binding.

Interestingly, in some cases the ligand binding properties of integrins have been shown to be cell type specific; for example, the $\alpha_2\beta_1$ integrin acts solely as a collagen receptor or both as a collagen and laminin receptor depending on the

cell type (Elices & Hemler, 1989; Languino et al, 1989). The reason for this cell type specific behaviour is not known, but based on our results one explanation could be that cell types with a higher integrin activation state (Van de Walle et al., 2005; Cruz et al., 2005) show lower ligand selectivity, and vice versa.

As discussed in section 1.3. divalent metal ions are essential for integrin function and the metal ion at the MIDAS site directly takes part in ligand binding. Accordingly, it was not a big surprise that ethylenediamine tetraacetate (EDTA), which chelates divalent metal ions, and a small molecular inhibitor L3008 (compound 2) described in paper III of this thesis (Käpylä et al., 2007), inhibited collagen and laminin binding to the α_1I and α_2I domain both in the closed and open conformations (Figures 5-7 in Tulla et al., 2008).

4.3. STUDIES ON THE EFFECTS OF SMALL MOLECULAR INHIBITORS OF COLLAGEN RECEPTOR INTEGRINS (Paper III: Käpylä et al., 2007)

Integrins have diverse and important roles in many biological processes and are involved in many human diseases. Accordingly, plenty of effort has been invested in studies aiming at developing integrin antagonists for the treatment of cardiovascular, inflammatory and metastatic diseases (see section 1.1.). Most of the drugs in clinical practice and drug trials that target integrins are antibodies, but there is an increasing interest towards developing small molecule inhibitors against integrins (Simmons, 2005). For the development of a specific small molecular drug, which is able to discriminate against one integrin out of the 23 other human integrins, one should choose a target subunit which exists only in one integrin. Fortunately, this is possible for all the integrins except for the two α_4 integrins, $\alpha_4\beta_1$ and $\alpha_4\beta_7$, and for the two α_V integrins, $\alpha_V\beta_1$, and $\alpha_V\beta_3$ (there are twelve β_1 integrins, two β_3 integrins, and two

β_7 integrins; Figure 2 in section 1.1.). Small molecular inhibitors that bind to β_2 have been developed (Gadek et al., 2002; Welzenbach et al., 2002), but these lack specificity as they recognize all of the four β_2 integrins (Figure 2). Nine of the 24 mammalian integrins (collagen-binding and leukocyte specific integrins) have an αI domain, which is responsible for the ECM ligand binding in that group of integrins (sections 1.1., 1.2.2., 1.3.2.). All these nine αI domains are unique and thus for all of them a specific small molecular inhibitor could in principle be developed, which recognizes specific structural features of the αI domain. One challenge in the development of specific drugs against integrins and αI domains is the highly dynamic structure of the extracellular region of integrins (see section 1.4.). The known small molecular αI domain inhibitors are allosteric inhibitors (Shimaoka & Springer, 2003; Simmons, 2005), which recognize and stabilize the closed conformation of the α_LI domain (BIRT0377, a member of a novel class of hydantoins — Last-Barney et al., 2001; Statins — Weitz-Schmidt et al., 2001) and α_2I domain (Arylamide compounds — Yin et al., 2006). Based on the results presented in this thesis, the closed αI domain is clearly a much better drug target than the open form as the closed form has a much higher ligand selectivity (Papers I-IV).

Based on the differences between the open and closed conformation of the α_2I domain (see section 1.4.; Emsley et al., 2000) we set two main structural criteria when searching for an effective α_2I inhibitor. The inhibitor should interact with the αC helix and with amino acids located just before the following α6 helix. Furthermore, it should coordinate with the Mg^{2+} ion at the MIDAS site so that the coordination state seen in the closed conformation is maintained upon inhibitor binding (Paper III: Käpylä et al., 2007). By docking a large number of molecules into the closed conformation of the α_2I domain, specific tetracyclic aromatic compounds, belonging to the group of polyketides (Thomas, 2001),

were identified as best fulfilling the two main structural criteria. Based on docking simulations these compounds coordinate to the MIDAS-Mg^{2+} ion, the aromatic rings of tetracyclic polyketides interact with Tyr285 of the αC helix, and the polar substituents of tetracyclic ring interact with the main-chain amino group of Glu256 (Fig. 1 in Käpylä et al, 2007). Furthermore, Leu296 seems to be important for the binding of these aromatic compounds.

The two most potent, pure inhibitors were compounds called **2** (methyl 2-ethyl-4,5,7,12-tetrahydroxy-6,11-dioxonaphthacenecarboxylate) and **3** (methyl 4,5,7, 12-tetrahydroxy-2(methylethyl)-6,11-dioxonaphthacenecarboxylate), which had IC_{50} values of about 50 μM and 10 μM, respectively, for the collagen I binding to the wild closed conformation of α_2I domain (Figures 1 and 3 in Käpylä et al., 2007). The IC_{50} value for collagen I binding to the open conformation (E318W mutant; see sections 4.1. and 4.2.) of the α_2I domain was about three times higher compared to the closed conformation indicating that the inhibitor inhibits both forms of the α_2I domain, but that the closed form is more sensitive to this inhibition.

At a concentration of ≥ 10 μM both compound **2** and **3** significantly inhibited cell adhesion to collagen I in transfected CHO cells having $\alpha_2\beta_1$ as their sole collagen receptor. Furthermore, CHO cells with the mutations Y285A and L296A in the α_2I domain could still bind to collagen I but were much less sensitive to inhibition by **2**, supporting the idea that Tyr285 and Leu296 are important for the inhibitor to bind to α_2I (Figure 3e in Käpylä et al., 2007). In addition to binding to the α_2I domain, compound **3** inhibited collagen binding to the α_1I, α_{10}I and α_{11}I domains despite their structural differences. This is not a surprise as the four αI domains of collagen receptor integrins bind collagens in a similar way (Zhang et al., 2003). Compound **2** had no effect on CHO cell

adhesion to fibronectin, which is the ligand for $\alpha_5\beta_1$ and α_V integrins (Danen et al., 2002). In summary, the potent small molecular inhibitors (**2** and **3**) identified in paper III seem to be specific for the collagen receptor subgroup of integrins. After paper III was published other small molecular inhibitors for collagen receptors have been developed (Nissinen et al., 2010; Koivunen et al., 2011), but more work is still required to discover a small molecular drug, which is able to specifically recognize only one of the four collagen-binding integrins.

4.4. COLLAGEN BINDING STUDIES OF THE WILD TYPE αI DOMAINS OF LEUKOCYTE INTEGRINS AND THEIR MUTANTS (Paper IV: Lahti et al., manuscript submitted for publication)

The physiological roles of collagen receptor and leukocyte specific integrins partially overlap. For example, $\alpha_2\beta_1$ integrin recognizes proteins that participate in the regulation of innate immunity (Zutter & Edelson, 2007), and leukocyte integrins bind collagens at least in some circumstances (Garnotel et al., 1995, 2000; Walzog et al., 1995). To make the first systematical comparison of human $\alpha_L\beta_2$ (lymphocyte function-associated antigen 1, LFA-1), $\alpha_M\beta_2$ (CR3) and $\alpha_X\beta_2$ (CR4) as collagen receptors, we produced the corresponding integrin αI domains both in the wild type and activated forms and measured their binding to collagen subtypes I-VI (see section 1.1.2.) and to the triple helical collagen analogue, GFOGER (see section 1.3.2.). The recombinant proteins were carefully designed to cover a comparable sequence in each αI domain. The α_LI, α_MI and α_XI domains were cloned by RT-PCR (reverse transcription polymerase chain reaction) from the total RNA from human white blood cells. The mutations I306G in α_LI (Huth et al., 2000), I316G in α_MI (Xiong et al., 2000) and I314G in α_XI (Vorup-Jensen et al., 2003) had been previously used to open the αI domain structure by inhibiting isoleucine side-chain binding to a

specific hydrophobic pocket. Furthermore, the α_LI domain had also been locked in the open conformation by creating an interchain disulphide bond (K287C/K294C) (Lu et al., 2001). These mutations were made and used also in this study. As a positive control for these collagen binding studies we used the wild type α_2I domain and its open mutant, E318W (see section 4.2.1).

The K_d value for the binding of the primary ligands, collagen I to the wild type α_2I and iC3b (Human inactivated complement fragment 3) to the α_XI domain were about 3 nM and 170 nM, respectively, whereas no significant binding of the wild type α_LI and α_MI domains was seen with ICAM-1 (Intercellular cell adhesion molecule-1) and iC3b. The activating mutations in α_2I, α_LI and α_MI increased binding to collagen I, ICAM-1 and iC3b, respectively. The I314G mutation in α_XI did not have any clear activating effect on iC3b binding (Figues 3-4 and Table 2 in Lahti et al., 2012). The wild type α_LI bound strongest to the basement membrane collagen IV and somewhat to the network-forming collagen VI, but not to the fibril forming collagens I-III and V. The activating mutations in α_LI mostly increased binding to the fibril forming collagens having only a small, if any, effect on collagen IV and VI binding. Accordingly, the selectivity towards different collagen subtypes was largely lost by the activating mutations of α_LI. Wild-type α_MI recognized all the tested collagen subtypes quite unselectively and the activation somewhat increased binding of all collagen subtypes. Wild-type α_XI also interacted with all collagen subtypes, preferring collagens IV and VI, but the activating mutation had only small effects on collagen binding, except for collagen IV, with which the effect was more pronounced (Figure 5 in Lahti et al., 2012). The strongest interactions were observed for the α_LI I306G:collagen I and α_XI I314G:collagen IV complexes with K_d values of about 80 nM and 180 nM, respectively. Furthermore, integrin α_XI (I314G) bound with high avidity ($K_d \approx 200$ nM) to

collagen VI (data not shown). Accordingly, collagen binding seems to be even stronger than is the binding of natural ligands to the α_LI, α_MI and α_XI domains using the same assay conditions (Figure 6 and Table 2 in Lahti et al., 2012).

Collagen receptor integrins recognize collagens based on the specific triple helical GFOGER-like sequences (see section 1.3.2.), and so we wanted to know whether the leukocyte integrins also recognize this same binding motif in collagens. As a positive control we showed that both the wild type and activated α_2I domain bind the GFOGER peptide very tightly with K_d values of 4 nM and 2 nM, respetively. Both wild type and I314G α_XI bound GFOGER with moderate affinity ($K_d \approx$ 200-300 nM), the activated α_MI domain bound the peptide weakly (Figure 7 and Table 2 in Lahti et al., 2012), but neither wild type nor the activated α_LI showed better binding than to the negative control, GST (not shown). To conclude, the leukocyte integrin α_XI recognizes the GFOGER peptide, whereas α_MI and α_LI may bind to different motifs than the actual collagen receptor integrins.

The role of leukocyte specific integrins as collagen receptors was also tested by cell adhesion assays, which showed that the integrin subunit α_X function blocking antibody markedly decreased adhesion of human promyelocytic leukemia cells to collagens I (not shown) and IV, antibodies for α_L showed some inhibition, whereas antibodies for the α_M subunit had no effect on binding (Figure 1 in Lahti et al., 2012).

The results presented in paper IV indicate that all three αI domains of leukocyte integrins are able to bind collagens and that the ligand preferences and the dependency on activation are different for each αI domain. Activation was shown to be a prerequisite for the α_LI domain to bind fibril forming collagens.

Similarly in paper II activation was shown to be a prerequisite for the binding of the collagen receptor α_2I domain to laminins (section 4.2.2.)

Possibly $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ act as collagen receptors in inflammatory cells before the "true" collagen receptor integrins appear and after that they may have an assisting role. This may be the case also for some cancers, such as leukemias, in which the levels of $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are elevated, as is seen from the Cancer Cell Line Encyclopedia (Barretina et al., 2012). Furthermore, as $\alpha_X\beta_2$ (CR4) binds to both the GFOGER motif and collagen IV quite tightly, it may have an overlapping function with the collagen receptor integrins.

5. CONCLUSIONS

In this work the structure and function of αI domains in collagen receptor and leukocyte specific integrins were studied with special emphasis on the effects of integrin activation in ligand selectivity. In paper I the three-dimensional structure for the activated form (C139S/E317A mutant) of the α_1I domain was solved and its ligand binding properties were analyzed. The E317A mutation was previously shown to activate the α_1I domain, whereas the C139S mutation made in this work turned out to be essential for producing enough pure protein for successful crystallizations and the subsequent X-ray analysis. The 3D structure for C139S/E317A was solved at a 1.9 Å resolution and it revealed a novel intermediate conformation, which had not been previously seen with any other αI domain.

Collagen receptor and leukocyte specific integrins are quite unspecific, each having both primary and secondary ligands. In this work the first systematic studies were performed for these two integrin groups to find out how integrin activation affects the binding and selectivity of both primary and secondary ligands. These kinds of studies are important not only for understanding the partially overlapping functions of integrins, but also for drug development in trying to identify specific small molecular inhibitors, which are able to discriminate between different integrin subgroups. Paper II dealt with collagen receptor integrins and paper IV with leukocyte specific integrins. In general, results from both of these papers clearly indicated that selectivity in ligand recognition is greatly reduced upon integrin activation. Conformational activation of both the α_1I and α_2I domain led to high-avidity binding to otherwise disfavoured collagen subtypes. Furthermore, the closed conformation of α_1I recognized the secondary ligands, laminin isoforms, significantly better than did α_2I. The α_1I E317A activating mutation not only increased laminin

binding but also reduced the selectivity between different laminins. Interestingly, the activating E318W mutation drastically increased binding of laminins -111, -211 and -511 to the α_2I domain, which only poorly recognized laminins in the wild type, closed conformation. These results clearly showed that activation was required for the α_2I domain to significantly bind laminins (Paper II). In paper IV all three αI domains of the leukocyte integrins tested were shown to bind collagens. The ligand preferences and dependency on activation was different for each αI domain. Activation was essential for the α_LI domain to bind fibril forming collagens. Possibly $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ act as collagen receptors in inflammatory cells before the "true" collagen receptor integrins appear and after that they may have an assisting role. Interestingly, in some cases the ligand binding properties of integrins have been shown to be cell type specific; for example, $\alpha_2\beta_1$ integrin acts solely as a collagen receptor or both as a collagen and laminin receptor depending on the cell type. The reason for this cell type specific behaviour is not known, but based on our results one explanation could be that cell types with higher integrin activation state have lower ligand selectivity, and vice versa.

Paper III, chronologically the first one, was focused on searching for an effective α_2I inhibitor. Based on the differences between the open and closed conformation of the α_2I domain two main structural criteria were set for this search. The inhibitor should interact with the αC helix and with amino acids located just before the following α6 helix. Furthermore, it should coordinate with the Mg^{2+} ion at the MIDAS site so that the coordination state seen in the closed conformation is maintained upon inhibitor binding. By docking a large number of molecules into the closed conformation of the α_2I domain specific tetracyclic aromatic compounds belonging to the group of polyketides were identified to best fulfill the two main structural criteria. By cell adhesion and

ligand binding studies two of these compounds were shown to be specific for the collagen receptor integrins.

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