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173

Mira Tulla

Collagen Receptor Integrins

Evolution, Ligand Binding Selectivity and the Effect of Activation

JYVÄSKYLÄN YLIOPISTO

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ABSTRACT

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Collagen receptor integrins: evolution, ligand binding selectivity and the effect of activation
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Yhteenveto: Kollageenireseptori-integriiniien evoluutio, ligandin sitomis-valikoivuus ja aktivaation vaikutus
Diss.

The collagen receptor integrins α1β1, α2β1, α10β1 and α11β1 belong to a subgroup of integrins with an inserted ligand binding αI domain. Here, the binding selectivities of the integrin α1I, α2I, α10I and α11I domains were elucidated. All αI domains had their own binding preferences. The ligand binding pattern of the integrin α10I domain was similar to the α1I domain; both domains favored non-fibrillar collagen types IV and VI over fibril-forming ones. The integrin α2I domain ligand pattern was the opposite. The collagen receptor αI domains mediated laminin binding as well, although with weaker avidity than for collagen. One residue; α1IR218, α2ID219 and α10IR218 in the corresponding αI domain, was found to have an important role in the determination of the ligand selectivity.

When integrins are activated the αI domain assumes a so-called open conformation. This conformation can be mimicked with a certain mutation. The constitutively active mutants of the α1I and α2I domains showed decreased selectivity towards collagens, although their binding to laminins was enhanced. The activation seems to be prerequisite for integrin α2I domain laminin binding.

Collagen receptor integrins have been considered as a vertebrate invention. However, recent genome sequencing of the tunicate species *Ciona intestinalis* revealed the presence of αI domain containing integrins. The *Ciona* α1I domain bound to collagen IX, but with a Mg-independent mechanism. The *Ciona* α1I domain did not recognize GFOGER or related sequences that are well-known binding motifs for vertebrate collagen receptor integrins. Moreover, the GFOGER motif was not found in *Ciona* collagens. The observation suggests that collagen receptor integrins may have evolved already in early chordates but the GFOGER based collagen binding mechanism is a later development.

Key words: integrin; αI domain; ligand selectivity; activation; evolution

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following scientific articles and manuscripts, which will be referred to in the text by their Roman numerals.

I Tulla, M., Pentikäinen, O.T., Viitasalo, T., Käpylä, J., Impola, U., Nykvist, P., Nissinen, L., Johnson, M.S. & Heino, J. 2001. Selective binding of collagen subtypes by integrin α1I, α2I, and α10I domains. J. Biol. Chem. 276: 48206-48212.

II Käpylä, J., Jäälinoja, J., Tulla, M., Ylöstalo, J., Nissinen, L., Viitasalo, T., Vehviläinen, P., Marjomäki, V., Nykvist, P., Säämänen, A-M., Farndale, R.W., Birk, D.E., Ala-Kokko, L. & Heino, J. 2004. The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix. J. Biol. Chem. 279: 51677-51687.

III Tulla, M., Lahti, M.J., Puranen, J.S., Brandt, A-M., Burcza, A., Käpylä, J., Salminen, T.A., Johnson, M.S. & Heino, J. 2006. Integrin activation decreases the selectivity of α2I domain for collagen subtypes but is prerequisite for laminin binding. Manuscript.

IV Tulla, M., Huhtala, M., Jäälinoja, J., Käpylä, J., Farndale, R.W. Ala-Kokko, L., Johnson, M.S. & Heino, J. 2006. Analysis of an ascidian integrin provides new insight into early evolution of collagen recognition. Manuscript.

RESPONSIBILITIES OF MIRA TULLA IN THE ARTICLES AND MANUSCRIPTS OF THIS THESIS

Article I:

I am mainly responsible for the production of the mutant and wild type integrin αI domains and the solid phase binding assays done for the study. However, for figure 1 Tiina Viitasalo produced the α10I-MPB fusion protein and performed the assays where it was used. Olli Pentikäinen from Mark Johnson's group at Åbo Akademi did the sequence analysis and molecular modelling. I wrote the article with Olli Pentikäinen, Jyrki Heino and Mark Johnson.

Article II:

I am responsible for the production of the integrin α10β1 expressing CHO cell line, the immunoprecipitations and cell spreading assays performed with it. In addition I've been involved in the production of the integrin αI domain mutants used in the study. However, the αI domain solid phase binding assays are performed by Jarmo Käpylä.

Manuscript III:

I am mainly responsible for the experimental work of the study. However, the solid phase binding assays of integrin α2I E318W mutant on collagen were performed by Anna Burcza. Matti Lahti performed the assay on figure 5B. Santeri Puranen from Mark Johnson's group did the molecular modelling. I wrote the article with Santeri Puranen, Jyrki Heino and Mark Johnson.

Manuscript IV:

I did the experimental work and Mikko Huhtala did the sequence alignments, phylogenetic trees and molecular models. I wrote the article with Mikko Huhtala, Jyrki Heino and Mark Johnson.

All the studies were performed under supervision of Professor Jyrki Heino.

ABBREVIATIONS

ADMIDAS	adjacent to MIDAS
αI domain	inserted domain of the α subunit in integrin
ATCC	American Type Culture Collection
BM	basement membrane
BSA	bovine serum albumin
βTD	β tail domain
CHO	Chinese hamster ovary
COL	collagenous domain
DDR	discoidin domain receptor
DxSxS	aspartate, any amino acid, serine, any amino acid, serine, a conserved sequence making up the MIDAS
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EM	electron microscopy
FACIT	fibril-associated collagen with interrupted triple helices
GPVI	glycoprotein VI
GST	glutathione-S-transferase
ICAM	intercellular adhesion molecule
I-EGF	integrin epidermal growth factor like domain
IG	immunoglobulin
K_d	dissociation constant
KHOS-240	human Caucasian osteosarcoma cell line
LG	laminin G-domain
LIMBS	ligand-associated metal-binding site
MBP	maltose-binding protein
MIDAS	metal ion-dependent adhesion site
MMP	matrix metalloproteinase
MULTIPLEXIN	multiple triple helix domains with interruptions
NC	non collagenous (non triple helical) domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSGL-1	P-selectin glycoprotein ligand-1
PSI	plexins, semaphorins, and integrins
RGD	arginine-glutamate-aspartate
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SAOS-2	human osteosarcoma cell line
SDS	sodium dodecyl sulfate
vWF	von Willebrand factor
vWFA	von Willebrand factor A domain
Å	Ångström, 1Å=0,1nm

1 INTRODUCTION

”Collagens are the most abundant proteins in a vertebrate body”. All researchers in biomedical fields have heard this many times and it is very appropriate as the first sentence of this thesis. Collagens are a group of extracellular matrix (ECM) proteins that contains around 30 members in humans. The topic here is the collagen receptor integrins, the cell surface molecules that mediate cellular interactions and attachment to collagen and ECM proteins. The universality and importance of not merely collagens, but in addition, the cellular systems developed for recognizing and communicating with them can readily be predicted from the first sentence.

Collagen receptor integrins are involved in many processes in normal and pathological physiology. Collagen receptor integrin α2β1, for example, has been shown to have a role in thrombosis and in cancer. Therapeutic agents targeted to collagen receptor integrins are under development. The basis for their development is the knowledge of collagen receptor integrin function on a molecular level revealed by basic research. The information produced here can be utilized in this kind of research.

This PhD dissertation is focused on collagen receptor integrin αI domain ligand recognition, the ligand binding mechanism, the determination of the ligand binding selectivity, the effect of integrin activation on ligand binding selectivity, and αI domain evolution. The thesis consists of four publications/manuscripts. The most important results of the studies are discussed with a focus on the work where the author’s contribution has been the most significant. The studies were mostly done in collaboration with a bioinformatics research group and the author’s involvement has been in the biochemical experimental studies. Therefore the integrin αI domain structural models presented in the publications and the manuscripts are not discussed in detail here. Integrin activation by clustering and signal transduction (so called outside-in signaling), although fundamental functions of integrins, are also out of the scope of this thesis and therefore are not covered.

2 REVIEW OF THE LITERATURE

2.1 Integrins

Integrins are the major family of cell adhesion molecules mediating cell-cell and cell-extracellular matrix (ECM) interactions (Hynes et al. 1987; Hynes 1992). The name "integrin" reflects the function of the molecules in integration of the cell actin cytoskeleton to the ECM. All metazoan organisms express these type I membrane proteins. Integrins are not merely gluing cells together or to the surroundings but also convey signals both in and out of cells and are crucial for cell survival and movement (Hynes 1992).

Integrins are composed of two distinct subunits, α and β, encoded by two separate gene families. Together they form a heterodimer with a larger extracellular part composed of a headpiece and two legs, a smaller cytoplasmic part and short transmembrane domains. Noncovalent interactions hold the subunits together. In vertebrates there are 18 α subunits and 8 β subunits that altogether form only 24 different heterodimers of the more than one hundred hypothetical ones.

Human integrins can be divided into four groups (Figure 1). RGD receptors recognize the RGD (arginine-glycine-aspartate) tripeptide motif in fibronectin and vitronectin. Laminin receptors are involved in adhesion to basement membranes (BM). Leukocyte integrins and collagen receptor integrins are both groups that contain an inserted (I) domain in their α subunits. However, two integrin heterodimers, namely α4β1 and α9β1 fall out of this grouping.

According to current knowledge, the αI domain containing integrins, whose subgroup; the collagen receptors, is the focus of this thesis, each contain only one type of β subunit. For the collagen receptor integrin α subunits α1, α2, α10 and α11 the pairing β subunit is β1. The other integrin heterodimers are presented in Figure 1.

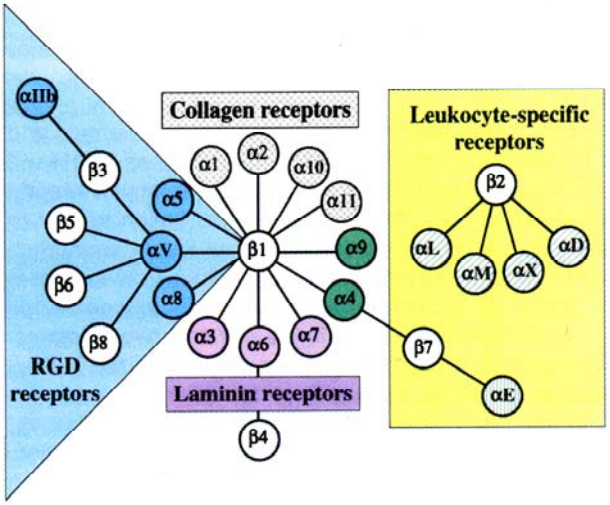

FIGURE 1 The mammalian integrin receptors. The possible αβ heterodimers are depicted and the subfamilies based on evolutionary relationships are color coded. Integrins with αI domains (gray stippling) as well as α4 and α9 (green) are found from chordates only. Laminin and RGD receptors are found from all metazoa. Figure is reprinted from Cell, Vol 110, Hynes, Integrins: bidirectional, allosteric signaling machines, 673-687, Copyright (2002), with permission from Elsevier.

2.1.1 Integrin structure in general

The first structure of an integrin ectodomain was published five years ago for a fibronectin receptor αVβ3 (Xiong et al. 2001). No equivalent structure for a collagen receptor integrin exists so far, but due to the high sequence similarity between integrins the collagen receptor structure is expected to be highly similar. The delay in obtaining the ectodomain structure stems from well known problems in the crystallization of membrane proteins that may be large in size and may have hydrophobic transmembrane sequences that make them poorly soluble. Part of the problem was overcome by truncation of the molecule removing the transmembrane segments.

The ovoid shaped globular headpiece of the integrin heterodimer is formed by the seven-bladed β-propeller domain of the α subunit and the βI-domain of the β subunit (called also the βA-domain or βI-like domain) (Figure 2). A subgroup of integrins contains an additional I "inserted" domain (αI domain or A domain) in the β-propeller domain of the α subunit. The βI and αI domains assume a similar Rossman (dinucleotide binding) fold, where a central β sheet is surrounded by α helices (Lee et al. 1995a; Xiong et al. 2001). A distinctive feature in the αI and βI domain is a metal coordination site called the metal ion-dependent adhesion site (MIDAS). Besides that the βI domain contains two additional metal binding sites, the ligand-associated metal-binding site (LIMBS) and ADMIDAS, which lies adjacent to a MIDAS and hence the name. The αI domain structure is discussed more thoroughly in the next chapter. The seven blades of the β-propeller are formed of repeats of an approximately 60 amino acid sequence that each fold into a four-stranded

antiparallel sheet. The domain contains four Ca^{2+}-ion binding sites that are solvent exposed and may be involved in allosteric regulation of the integrin ligand binding (Xiong et al. 2001). Besides integrins, the β-propeller is a broadly used fold in molecules involved in molecular interactions (Cioci et al. 2006).

The α subunit leg that attaches the headpiece to the plasma membrane is composed of three β-sandwich domains; an immunoglobulin (Ig)-like thigh domain and calf-1 and calf-2 domains (Figure 2). Between thigh and calf domains there is a flexible linker, knee or "genu," where a Ca^{2+}-ion is coordinated.

The β subunit leg is composed of a hybrid domain similar to the I-set Ig domains, PSI (plexins, semaphorins and integrins) domain, four cysteine-rich epidermal growth factor (EGF) domains 1-4 and a β tail domain (βTD) (Xiong et al. 2001; Beglova et al. 2002).

By homology based molecular modeling it has been predicted that the general structure of the ectodomains of collagen receptor integrins is similar to the solved αVβ3 structure, the most striking difference being the additional αI, inserted domain, that these receptors contain (White et al. 2004).

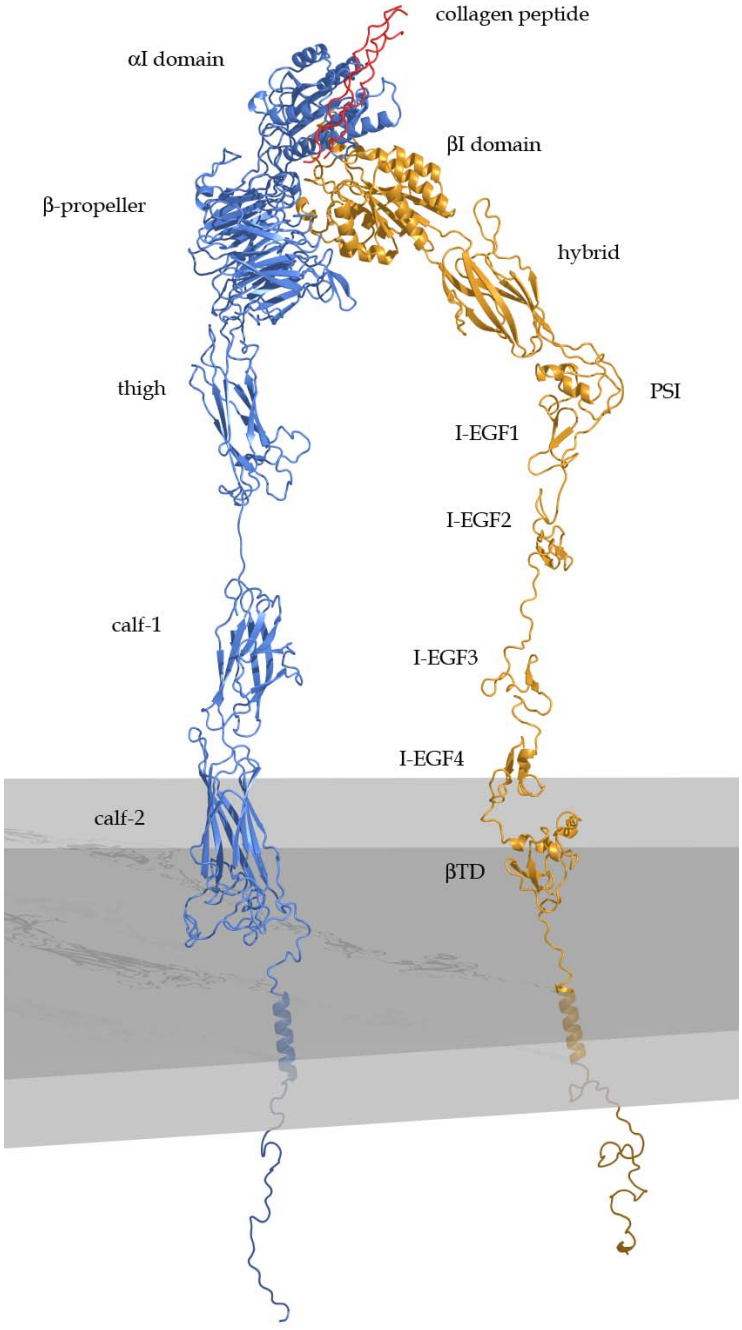

FIGURE 2 Integrin α2β1 heterodimer. Extended form with open headpiece and bound collagen peptide (red). The α subunit consists of an αI domain, β-propeller, thigh and two calf domains. The β subunit consists of a βI domain, hybrid domain, PSI (plexins, semaphorins and integrins) domain, four I-EGF (epidermal growth factor) domains and a βTD (β tail domain). The transmembrane and cytoplasmic domains of both α and β subunits are unlabeled. Theoretical model of integrin α2β1 by Mikko Huhtala based on several crystal structures and cryo electron microscopy reconstructions (Emsley et al. 2000; Xiong et al. 2001 & Xiao et al. 2004).

2.1.2 Collagen receptor integrin αI domain structure

All collagen receptor integrins, namely α1β1, α2β1, α10β1 and α11β1 belong to the subgroup of αI domain containing integrins (Takada et al. 1988; Briesewitz et al. 1993; Camper et al. 1998; Velling et al. 1999). The inserted, independently folding αI domain at the N-terminus of the α subunit mediates ligand binding (Tuckwell et al. 1995; Calderwood et al. 1997; Dickeson and Santoro 1998). The αI domains have been crystallized from α1 (Nolte et al. 1999; Rich et al. 1999; Salminen et al. 1999, Nymalm et al. 2004), α2 (Emsley et al. 1997, 2000), αL (Qu and Leahy 1995) and αM (Lee et al. 1995a, b) subunits and illustrated to assume a structure called a Rossman fold or dinucleotide binding fold, where a mostly parallel central β sheet is surrounded by α helices. Other protein domains adopting the same fold are found for example in the small G proteins, the von Willebrand factor A domain (Bienkowska et al. 1997; Huizinga et al. 1997), collagen VI and the complement factor B (see reviews by Tuckwell 1999 and Whittaker and Hynes 2002).

On top of the αI domains resides a well-conserved cation binding site, MIDAS, that is formed by three loops of the domain coordinating a metal ion. MIDAS has been recognized as the major, if not only, ligand binding site in integrins possessing the αI domain and hence the ligand binding is cation dependent (Michishita et al. 1993; Lee et al. 1995b). A conserved sequence DxSxS and an additional threonine residue (T221, α2I numbering) form the site. When a ligand is bound, aspartate (D151) makes a water-mediated bond to the cation, and both serines (S153, and S155) and the threonine (T221) bond directly through their hydroxyl oxygens (Figure 3). Two acidic residues (D254 and E256) make water mediated bonds to the cation. A glutamate residue from collagen can coordinate the metal directly and two water molecules complete the coordination. The cation on the MIDAS can either be Mg^{2+} or Mn^{2+}, but Ca^{2+} has been shown to be too big to fit in to the site (Emsley et al. 2000). Metal coordination to the unliganded domain is slightly different.

Specific to the collagen binding integrin αI domains is the existence of an additional α helix, termed the αC-helix, on the top face of the domain. The helix helps in forming a groove on the top surface of the domain. It was suggested that the αC-helix would form a binding pocket that maximizes the interactions with a rod like collagen. However, the crystal structure of the integrin α2I domain in complex with a collagenous triple helical peptide revealed that only the edge of the groove was utilized in collagen binding and showed no involvement of the αC-helix. Nonetheless it has been speculated that the αC-helix might form interactions with a bundle of numerous collagen triple helices; the collagen fiber, and that it might have a role in preventing unspecific interactions with collagens (Emsley et al. 2000, 2004).

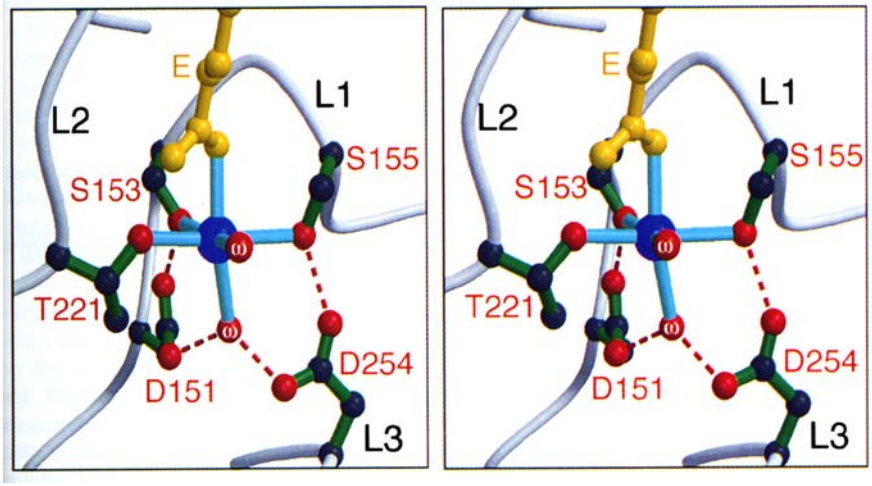

FIGURE 3 Stereo image of the integrin α2I domain MIDAS with bound collagen peptide. The metal ion is represented as a blue ball. The side chains coordinating the metal are shown as ball-and-stick. Oxygen atoms are red, carbon is black. Water molecules are labeled with "ω". The collagen glutamate is yellow. The three loops (L1-L3) of the upper face of αI domain are shown as gray ribbons. Reprinted from Cell, 101, Emsley et al., Structural basis of collagen recognition by integrin α2β1, 47-56, Copyright (2000), with permission from Elsevier.

2.2 Integrin activity modulation by conformational changes

The utmost importance of integrin activation is often elucidated with an example of platelet integrins that have a role in the arrest of bleeding and in the formation of a blood clot. Mistimed hemostasis or the body's failure to launch the process could be fatal. Therefore there need to be mechanisms to activate integrin ligand binding at the right place in a timely manner and to keep them inactive when their action is not needed. Regulation is achieved by conformational changes that propagate through domains from cytoplasmic parts to extracellular ones (inside-out), or ligand binding can induce changes in the αI domain, in the case of αI domain containing integrins, that cause the separation of transmembrane and cytoplasmic parts and initiate signaling cascades inside the cells (outside-in).

2.2.1 Conformational changes in integrin αI domain

Insight into integrin activation was provided by the integrin αI domain crystal structures that revealed two different conformations, closed and open, that differed by the nature of the metal ion (Lee et al. 1995a, b). The MIDAS of the open conformation was coordinated by a glutamate residue from the adjacent domain in the crystal lattice. The α2I domain structure in complex with a collagenous peptide that was resolve later proved the two conformations relevant (Emsley et al. 2000). The opening of the domain causes a significant

rise in affinity (Shimaoka et al. 2001) and therefore the open and closed conformations have also been referred to as high-affinity conformation and low-affinity conformation, respectively. It has been assumed that the two conformations, open and closed, exist in dynamic equilibrium, where the closed state may be favored in resting cells (Li et al. 1998; Shimaoka et al. 2001). For some integrin αI domains, a third conformational state, an "intermediate" conformer, is also predicted to exist, but for the α1I and α2I domains it does not seem probable (Jin et al. 2004).

Integrin αI domain activation is gained through allostery. The αI domain conformational activation includes rearrangements of the domain: changes in the MIDAS ion coordination that move the metal ion 2 Ångströms (Å), movements of the helices such as a small movement of the α1 helix, unwinding of the αC-helix and a downward movement of the C-terminal helix α7 by 10 Å that is considered the key feature of the process.

The two I domains of the integrin heterodimer α and β subunits seem to be able to affect each others conformation with a fascinating mechanism. The activation of the αI domain may be evoked by pulling down the α7 helix, which is suggested to be performed by the βI domain (Yang et al. 2004) (Figure 4). A conserved glutamate at the linker region in between the αI domain and the β propeller presumably works as an "intrinsic ligand" for the βI domain MIDAS. When the βI MIDAS is activated it may bind the glutamate of the linker. The formation of this intersubunit bond may exert a pull on the αI domain's α7 helix and thereby activate the αI domain (Lu et al. 2001; Alonso et al. 2002; Jin et al. 2004; Yang et al. 2004).

2.2.2 Conformational changes in integrin heterodimer during activation (inside-out)

Three overall conformations for integrin heterodimers have been detected using the first crystal structure of an integrin ectodomain (αVβ3) and by electron microscopic (EM) imaging of αVβ3 and αIIbβ3. It has been predicted that the conformational changes during activation are general for all integrins (Xiong et al. 2001; Takagi et al. 2002, 2003; Xiao et al. 2004).

In the crystal structure the heterodimeric integrin was observed to assume a bent conformation where the headpiece was brought close to the legs and plasma membrane, seemingly unavailable for ligand binding (Figure 4). It was assumed that the bent state probably would not exist on the cell surface but could rather be an artifact of the crystallization procedures. Later studies have confirmed that the bent state is physiologically relevant and represents the inactive conformation (Beglova et al. 2002; Takagi et al. 2002). The bent conformation may be unable to bind biological ligands, although some peptide antagonists are capable of binding to it (Xiong et al. 2002; Takagi et al. 2002). The integrin cytoplasmic domain association, although apparently with weak interactions only, has been speculated to stabilize integrins in the inactive bent state (Lu et al. 2001; Vinogradova et al. 2002, 2004). Inside-out signaling induced

separation of the cytoplasmic tails leads to extension of the integrin extracellular part. This has been described to take place in a switchblade type of motion at the "genu" or knee of the integrin subunits (Beglova et al. 2002; Takagi et al. 2002). A conformation, where the heterodimer assumes the extended conformation but the headpiece still remains closed and in a low affinity state precedes the fully activated extended conformer with an open headpiece (Shimaoka et al. 2002; Takagi et al. 2002). In the closed headpiece conformer the angle between the βI domain and the hybrid domain is more acute than in the open headpiece conformer. Supposedly the hybrid domain swings out from the βI domain due to disruption of the subunit interface leading to an open headpiece. This motion may evoke the downward movement of the α7 helix in the βI domain that may be coupled to MIDAS reorganization and activation of the domain with the same basic mechanism as described above for αI domain (Luo et al. 2004a; Xiao et al. 2004). The activated βI MIDAS may then bind the glutamate residue from the α subunit (the "intrinsic ligand") and this interaction may activate the αI domain and ligand binding (Alonso et al. 2002; Takagi & Springer 2002; Yang et al. 2004).

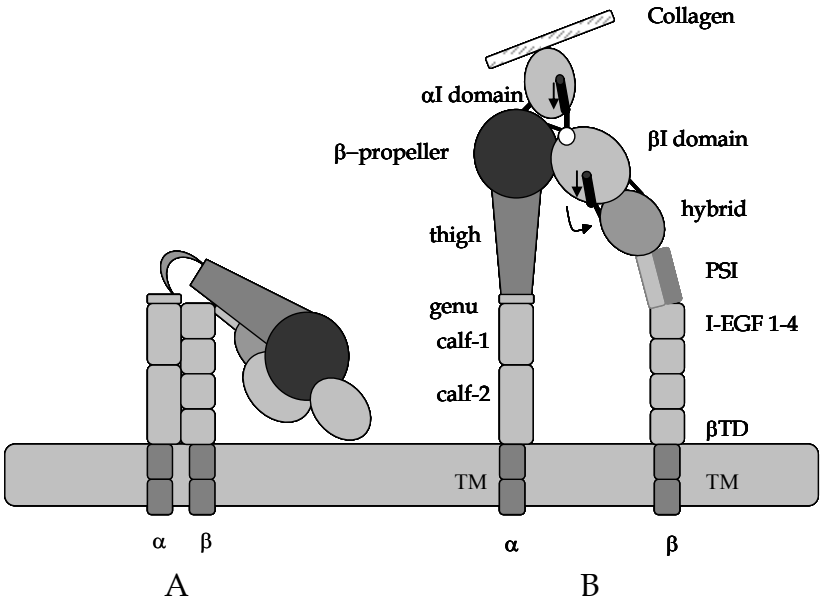

FIGURE 4 The two extreme conformations of the integrin heterodimer. The "bent" low affinity conformation (a) and the high affinity "extended" conformation with open headpiece and bound ligand (b). The movement of the α7 helix in the αI and βI domains is shown with straight arrows. Swing-out of the hybrid domain is represented with a bent arrow. The "intrinsic ligand" glutamate is represented with a white ball. Figure is based on Luo & Springer 2006.

2.2.3 Integrin activating factors

A key player in integrin activation on the cytoplasmic side is believed to be talin, an actin binding protein that links integrins to the actin cytoskeleton (Horwitz et al. 1986). The so-called inside-out activation of integrins can be triggered by a talin molecule headpiece binding to the cytoplasmic domain of the β subunit (Calderwood et al. 1999, 2002; Vinogradova et al. 2002; Tadokoro et al. 2003). This binding leads to separation of the α and β cytoplasmic and transmembrane domains from each other and to integrin activation (Vinogradova et al. 2002; Luo et al. 2004b).

Metal ions are known to regulate the affinity of integrins. In full heterodimers there are several divalent cation binding sites in both α and β subunits. Calcium binding sites are found on the β propeller domain of the α subunit, and in the βI domain there are three divalent cation binding sites: LIMBS, MIDAS, and ADMIDAS (Xiong et al. 2001). The heterodimeric ectodomains in the extended high-affinity conformers have been detected in solutions containing Mn^{2+}, while the low affinity conformation has been obtained in the presence of Ca^{2+} ions alone (Xiong et al 2001; Takagi et al. 2002). Generally speaking, magnesium and manganese ions have been observed to enhance ligand binding and calcium ions to inhibit it. LIMBS in the β subunit has been seen as a positive regulator of ligand binding to MIDAS, and ADMIDAS as a negative regulator (Chen et al. 2003). The explanation for LIMBS mediating positive regulation could be as suggested, based on molecular dynamics studies on the tripeptide RGD binding to the βI domain MIDAS, a direct coordination of the RGD ligand motif to the LIMBS ion (Craig et al. 2004).

Integrins are known to intercommunicate, meaning that they can activate or inhibit each other (Schwartz and Ginsberg 2002; Hynes 2002). For example integrin α2β1 signaling in platelets may cause integrin αIIbβ3 activation (Hynes 2002). Other receptors such as G-protein coupled receptors are also known to activate integrins.

2.2.4 Force in integrin activity and avidity regulation

Force, such as shear force in arteries or the mechanical force exerted by the cytoskeleton to molecules coupled to it, may affect the lifetime of molecular complexes. One might expect the lifetime of a bond to shorten by applying force to it, which is the case with most of the bonds. The term "slip bond" refers to the cases where dissociation is accelerated by force. However, the opposite behavior has been encountered in some particular molecular interactions. "Catch bonds", bonds whose lifetime is prolonged by force (Dembo et al. 1988) were experimentally demonstrated to exist for the first time only recently for P-selectin interacting with the P-selectin glycoprotein ligand 1 (PSGL-1) (Marshall et al. 2003). Since then the phenomenon has been documented in other systems as well; *Escherichia coli* fimbriae lectin-like adhesion protein FimH binding to mannose (Thomas et al. 2002, 2004), an actomyosin bond (Guo and Guilford 2006) as well as

for L-selectins (Sarangapani et al. 2004; Yago et al. 2004). It has been proposed that other molecules too could be capable of similar behavior. Promising candidates are the integrin family of cell adhesion receptors that form force-sustaining adhesion sites where the cell's actin cytoskeleton is coupled to the ECM molecules.

It is known that integrin αI domains undergo conformational changes that regulate ligand binding affinity. The conformational changes in the αI domain include a downward movement of the α7 helix leading to rearrangements of the metal-ion coordination on MIDAS and a subsequent increase in affinity (Shimaoka et al. 2001). Molecular dynamic simulations have suggested that applied force could also carry out the task of pulling down the α7 helix (Jin et al. 2004) and hence lead to increased affinity and bond lifetimes (catch bonds) in the αI domain. The same could be true at the level of a whole integrin as well, where large conformational changes lead to the activation of the heterodimer and, in addition, could lead to prolonged bond lifetimes (Zhu et al. 2005). Yet these hypotheses remain to be experimentally proven. Whether or not catch bonds exist on integrins these molecules already have one mechanism for forming force sustaining adhesions. That is the formation of clusters, where a group of integrins forms bonds whose strength is determined by the number of integrins.

2.3 Collagen receptor function

Collagen-integrin interactions have important roles in key physiological states like cell growth, adhesion, migration, differentiation, ECM assembly and angiogenesis as well as in pathological states such as thrombosis and tumor metastasis (Hynes 1992; Senger et al. 1997). The collagen receptor integrin α2β1 is known to function as a receptor for Echovirus-1 as well (Bergelson et al. 1992). Collagen receptor integrins α1β1, α2β1 and α11β1 are able to reorganize the collagenous matrix in a process called collagen contraction that is especially important during wound healing (Klein et al. 1991; Gotwals et al. 1996; Tiger et al. 2001). Collagen synthesis as well is regulated through these receptors (Langholz et al. 1995; Riikonen et al. 1995; Gardner et al. 1999).

Integrins α1β1 and α2β1 show the broadest tissue expression pattern of collagen receptors during embryonic development (Wu & Santoro 1994; Gardner et al. 1996). In adults, integrin α1β1 expression is encountered on cells of mesenchymal origin, like fibroblasts and smooth muscle cells and on endothelial tissues (Voigt et al. 1995). The integrin α2 chain is expressed on fibroblasts, endothelial cells, and epithelial cells, in addition to being the sole collagen receptor integrin in platelets. In contrast, integrins α10β1 and α11β1 show more restricted expression patterns. Integrin α10β1 expression appears to be mesenchyme-specific and is encountered in cartilage, lung, heart, trachea, aorta and spinal chord (Camper et al. 2001). During embryonic development integrin α11β1 expression was the strongest around forming cartilage (Tiger et al. 2001).

2.3.1 Collagen receptor knock-outs

Insights into protein function during development have been traditionally obtained from knock-out mouse models. Therefore knock-outs for all collagen receptor integrin α subunits have been developed. Surprisingly, relatively mild phenotypes have been described for all of these; embryonic development has not been impaired and the mice have been viable in all cases.

Integrin α1 knock-out mice had defects in cell proliferation, angiogenesis and in regulation of collagen synthesis in skin fibroblasts (Gardner et al. 1996, 1999; Pozzi et al. 1998, 2000). When bone regeneration after fracture was studied the α1-knock out mice developed considerably less callus tissue and a defect in cartilage formation was detected (Ekholm et al. 2002). Accelerated aging-dependent development of osteoarthritis has been detected in integrin α1 deficient mice (Zemmyo et al. 2003). Additionally, after glomerular injury the lack of integrin α1β1 has been shown to lead to glomerulosclerosis, where glomerular tissue is replaced by ECM (Chen et al. 2004).

In integrin α2-deficient mice, platelet adhesion to collagen I was abolished and the animals developed some abnormalities in mammary gland branching morphogenesis (Chen et al. 2002; Holtkötter et al. 2002). In experiments *in vitro* integrin α2β1 was required for keratinocyte adhesion to collagen, but not for fibroblasts where other collagen receptor integrins seem to compensate (Zhang et al. 2006). In α2-null mice wound healing was normal and integrin α2β1 was not needed in re-epithelialization (Chen et al. 2002; Grenache et al. 2006). A recent article reported an increase in neoangiogenesis in the wound microenvironment due to an α2-deficiency (Grenache et al. 2006). During acute peritonitis the knock-out mice were seen to have defects in innate immunity due to defects in mast cell function (Edelson et al. 2004).

Integrin α10 absence led to retarded growth of long bones due to defects in the growth plate. Otherwise the mice were fertile and the life span was the same as for wild type mice (Bengtsson et al. 2005). Integrin α11 deficiency does not hinder embryonic development but may lead to dwarfism (Tiger 2002).

Meanwhile, as expected, the genetic ablation of the integrin β1 subunit, which pairs with 12 different α subunits, led to embryonic lethality (Fässler and Meyer 1995; Stephens et al. 1995). Collagen receptor integrins have a tremendous capacity to compensate for each other due to the overlapping ligand binding patterns that may account for the mild phenotypes seen for α subunit knock-outs. Probably, to be able to see bigger differences, double or triple collagen binding integrin knock-outs are needed. So far, however, these have not been reported.

2.3.2. Collagen receptor integrin ligand binding

Collagen receptor integrins, like other integrins, are known to bind to several ligands. The ligand binding patterns of collagen receptors are overlapping and may include other ECM molecules, like laminins and tenascin as well. The well

known and ubiquitous ECM glycoprotein fibronectin, however, is not one of the ligands for collagen receptors.

2.3.2.1 Collagen binding

2.3.2.1.1 The collagen superfamily

Collagens are a very diverse group of around thirty members in humans but all metazoans are known to express them (Boot-Handford et al. 2003). They are formed of three polypeptide strands, the α chains, which coil into a triple helix. Characteristic to all collagens is the presence of a minimum of one triple helical collagenous domain (COL) containing a Gly-x-y (x is often proline, and y 4-hydroxyproline) repeat in the polypeptides. Hydroxylated amino acid residues, 3- and 4-hydroxyprolines and hydroxylysines are formed by post-translational modifications. Collagens of the fibrillar subgroup consist merely of a large continuous triple helical sequence, but in other subgroups the molecules may have interruptions and/or globular domains (NC, non triple helical domains) as well. Collagen self association creates the fibers and other supramolecular complexes. Collagens are grouped into subgroups according to sequence similarities and the supramolecular complexes they form (Prockop and Kivirikko 1995; Myllyharju and Kivirikko 2004; Ricard-Blum and Ruggiero 2005) (Table 1).

Fibrillar collagens, also considered as the classical collagens, are known in almost all multicellular organisms. However, during evolution, arthropods and nematodes have lost fibrillar collagens (Boot-Handford and Tuckwell 2003). In vertebrates, collagen I is the most abundant protein. The group of fibrillar collagens is comprised of the more common collagen types I, II and III, the minor types V and XI, and the latest additions to the group: XXIV and XXVII (Ricard-Blum and Ruggiero 2005). These collagens form heterotypic fibrils where both fibrillar and collagens from other subgroups may be mixed. Fibrillar collagens are secreted from the cells as procollagens, precursors that in most cases are processed by clipping the N- and C-terminal non-collagenous propeptides. Propeptide removal initiates collagen fibrillogenesis (Prockop and Kivirikko 1995). Characteristic to the collagen fibers is a certain 67nm periodicity that is also called D-banding.

Fibril-associated collagens with interruptions in the triple helix (FACIT) are the largest subgroup among collagens. Collagens that belong to this group include IX, XII, XIV, XVI and the new additions to the collagen family; XIX, XX, XXI and XXII (Myllyharju and Kivirikko 2004). Collagen IX is found covalently associated to the surface of the cartilage collagen type II fibers, where it supposedly mediates interactions with other matrix molecules (van der Rest and Mayne 1988; Ricard-Blum et al. 2000). A collagen IX like FACIT collagen has been cloned from a basal chordate *Ciona intestinalis* and shown to exist in the squid *Sepia officinalis* as well (Rigo et al. 2002; Vizzini et al. 2002).

Collagen subtypes, their expression sites and some elucidation of their most important functions and/or involvement in collagen supramolecular complexes are listed in Table 1.

TABLE 1

Group	Collagen type	Main tissue expression sites in vertebrates	Function	Reference
Fibrillar collagens	I	Most connective tissues, bone, tendon, skin	Forms fibers giving tensile strength to connective tissues	Prockop and Kivirikko 1995 (review)
	II	Cartilage specific, major collagen type in cartilage,vitreous of the eye	Constitutes the core of a collagen fibril	Cremer et al. 1998 (review)
	III	Skin, lung, blood vessels	Forms fibrils in elastic tissues	Prockop and Kivirikko 1995 (review)
	V	Wide distribution in non-cartilaginous tissues, lung, cornea, bone	Regulates collagen fibril diameter	Birk et al. 1990
	XI	Cartilage specific, eye	Regulates collagen fibril diameter	Cremer et al. 1998 (review)
	XXIV	Developing bone and eye	Not known, may regulate collagen fibril diameter	Koch et al. 2003
	XXVII	Chondrocytes, variety of epithelial cell layers in developing tissues	Not known, may heterotrimerize with collagen XXIV, may associate with basement membrane (BM) under epithelial cells	Boot-Handford et al. 2003; Pace et al. 2003
Network forming collagens	IV	BMs	Component of BMs	Kühn 1995 (review)
	VIII	Various tissues, BMs like Descemet's membrane	Forms hexagonal lattices, may take part in angiogenesis, tissue remodeling and fibrosis	Prockop and Kivirikko 1995 (review); Ricard-Blum et al. 2000 (review)
	X	Hypertrophic cartilage	Forms fine filaments in cartilage, hexagonal lattices	Kielty et al. 1985; Prockop and Kivirikko 1995 (review); Cremer et al. 1998 (review); Ricard-Blum et al. 2000 (review)

Continues.

Beaded filament forming collagen	VI	Major component of ECM of most tissues	Forms microfibrillar networks that link cells to ECM	Poole et al. 1988; Lampe and Bushby 2005 (review)
Anchoring fibril forming collagen	VII	Dermo-epidermal junction	Links BMs to anchoring plaques	Prockop and Kivirikko 1995 (review); Ricard-Blum & Ruggiero 2005 (review)
FACIT collagens	IX	Cartilage specific	Mediates interactions with proteoglycan macromolecules, attached to the surface of collagen II fibrils	Olsen 1997 (review); Prockop and Kivirikko 1995 (review); Cremer et al. 1998 (review)
	XII	Areas of high mechanical stress; tendons, ligaments, perichondrium and periosteum	Associated with the surface of collagen fibrils, regulates collagen I fibril diameter, may function as a "shock absorber" between collagen fibrils	Ricard-Blum et al. 2000 (review); Gelse et al. 2003 (review)
	XIV	Various tissues rich in collagen I	Organization of collagen fibrils	Ricard-Blum et al. 2000 (review); Gelse et al. 2003 (review)
	XVI	Various tissues, skin, cartilage	May have a role in anchoring microfibrils to BM	Lai & Chu 1996; Kassner et al. 2003; Ricard-Blum et al. 2000 (review)
	XIX	Skeletal muscle, spleen, prostate, kidney, liver, placenta, colon and skin, present in BM zone	Not shown to associate with fibrils, may be involved in the assembly of embryonic tissues and maintenance of some adult tissues	Myers et al. 2003; Ricard-Blum et al. 2000 (review)
	XX	Corneal epithelium, skin, cartilage and tendon	Possibly attached to the surface of collagen fibrils	Koch et al. 2001; Myllyharju and Kivirikko 2004 (review)
	XXI	Heart, stomach, kidney, skeletal muscle, placenta	Possibly interacts with other collagens	Fitzgerald and Bateman 2001; Myllyharju and Kivirikko 2004 (review)
	XXII	At tissue junctions		Koch et al. 2004

Continues.

Transmembrane collagens	XIII	Many tissues; epithelial, mesenchymal, neural tissues	Can be a component of focal adhesions	Pihlajaniemi et al. 1987; Prockop and Kivirikko 1995 (review); Hägg et al. 1998; Franzke et al. 2005 (review)
	XVII	Skin	Hemidesmosomal protein	Diaz et al. 1990; Giudice et al. 1993; Prockop and Kivirikko 1995 (review); Myllyharju & Kivirikko 2004 (review); Franzke et al. 2005 (review)
	XXIII	Metastatic tumor cells, lung, cornea, brain, skin, tendon, and kidney		Banyard et al. 2003; Franzke et al. 2005 (review); Koch et al. 2006
	XXV	Cerebral neurons	Stabilize aggregates of amyloid β-peptide	Hashimoto et al. 2002; Franzke et al. 2005 (review)
Multiple triple helix domains with interruptions (Multiplexin)	XV	Adrenal gland, kidney, and pancreas	May form networks of structural importance for the integrity of BMs	Myers et al. 1992; Muragaki et al. 1994; Ricard-Blum & Ruggiero 2005 (review)
	XVIII	Liver, kidney, placenta	May form networks of structural importance for the integrity of BMs	Rehn & Pihlajaniemi 1995
Collagens not grouped yet	XXVI	Testis and ovary during development	Does not interact with collagen fibrils	Sato et al. 2002; Ricard-Blum & Ruggiero 2005 (review)
	XXVIII			Koch et al. 2004

2.3.2.1.2 High affinity collagen recognition motifs and the ligand binding mechanism of integrin αI domains

The triple helical conformation of collagen is essential for recognition by integrins, as denatured collagen cannot be bound by these receptors (Morton et al. 1994). Of the post-translational modifications it is known that proline 4-hydroxylation of collagen I is needed for integrin α1β1 but not for α2β1 binding (Perret et al. 2003). Fibril-forming and type IV collagens have been reported to contain several binding sites for collagen receptor integrins α1β1 and α2β1 with a broad spectrum of binding affinities (Rich et al. 1999).

Collagenous ligands bind to a groove on top of the αI domains. The interactions with collagen form between the residues of three loops of the αI domain that make up the ligand binding site MIDAS (Figure 2). A collagenous hexapeptide GFOGER (O is 4-hydroxyproline) is the best known high affinity binding sequence in fibril-forming collagen subtypes and in network forming collagen IV for the α1I, α2I and α11I domains (Knight et al. 2000; Xu et al. 2000; Zhang et al. 2003). According to the crystal structure of the collagenous GFOGER peptide in complex with the α2I domain the majority of the interactions form with one collagen chain only. A glutamate residue from the GFOGER sequence coordinates the metal directly and the arginine residue makes a salt bridge to D219. Hydrophobic interactions are formed with α2I domain residues Y157 and L286, and hydrogen bonds to residues N154, Y157 and H258 (Emsley et al. 2000).

It has been shown that no prior activation of the αI domain is needed for GFOGER recognition (Siljander et al. 2004). However, the repertoire of amino acid recognition sequences rises, when the integrin αI domain is activated, to include sequences such as GLOGER, GMOGER, GLSGER and GASGER (Emsley et al. 2000; Knight et al. 2000; Xu et al. 2000; Siljander et al. 2004). Quite recently it was found out that motifs GROGER and GAOGER also function as integrin α1β1 and α2β1 binding sites, of which the former represents a high affinity binding motif. The GROGER sequence may in fact mediate higher affinity binding of the α2I domain than GFOGER and GLOGER sequences (Kim et al. 2005). It is readily seen from the list of binding motifs that most of them contain the common theme GxOGER. A strict requirement for the motif is the existence of a glutamate (E) in it (Emsley et al. 2000). These types of motifs are especially abundant in fibril-forming collagens. A shared feature in ligand binding of I domain containing integrins is that the octahedral metal ion coordination is completed with an acidic residue from the ligand.

Distinct binding sites for integrins α1β1 and α2β1 in collagen IV have been detected (Tuckwell et al. 1995; Calderwood et al. 1997). For integrin α1β1 a recognition site on collagen IV was described where three amino acid residues (R, D, and D) in three different chains of the collagen heterotrimer comprise the binding site (Golbik et al. 2000). In collagen XIII, which is recognized by α1β1 but does not contain GFOGER, some other motif has to function as the integrin α1β1 binding site (Nykvist et al. 2000).

Cells have been shown to transmit mechanical forces to the ECM by using integrins that can partially unfold proteins like fibronectin (Baneyx et al. 2002). ECM molecules, such as collagen and fibronectin, contain recognition sites that are exposed only by conformational changes such as force-induced stretching of the molecule or by proteolytic processing by matrix metalloproteinases (MMP), but otherwise stay buried inside the structure. These kinds of sites are called cryptic. It has been suspected that the occurrence of cryptic sites is a rather common phenomenon in ECM molecules (Schenk & Quaranta 2003). On the other hand force induced stretching of a molecule may also destroy and reduce the number of binding sites for a given receptor. Collagen contains cryptic tripeptide RGD motifs that can be recognized by α3β1, α5β1, αVβ3 and possibly by αIIbβ3 integrins, which do not normally bind native collagen. For the binding to occur, collagen needs to be cleaved and degraded to single strands. For collagen receptor integrins there is no evidence of cryptic sites on ECM molecules. On the other hand it is known that denatured collagen is not recognized by these receptors as the triple helical conformation is crucial.

2.3.2.2 Laminin as a ligand for collagen receptor integrins

Basement membranes (BMs) are a very ancient form of ECM. They are the structures that help in compartmentalization of tissues. The heterotrimeric ECM glycoprotein laminin is a key component in BMs. The laminin superfamily of proteins includes several different isoforms whose expression differs both spatially and temporally. The currently known 5 α, 3 β and 3 γ laminin chains form altogether over 15 cross- or T-shaped trimers with coiled-coil central domains. The nomenclature for laminins was renewed recently and according to the new system the names consist now of the corresponding α, β and γ chain numbers (Aumailley et al. 2005). Hence, the prototype laminin earlier known as laminin-1 is now called laminin-111 (α1β1γ1).

Laminin-111 is the best characterized laminin and is expressed for example in epithelial BMs (Ekblom et al. 2003). It is the major laminin expressed during early embryogenesis (Colognato & Yurchenco 2000). Laminin-211 (earlier known as laminin-2 or merosin) is expressed in skeletal muscle and peripheral nerves (Leivo and Engvall 1988; Patton et al. 1997). The absence of or abnormalities in the laminin α2 chain are associated with congenital muscular dystrophies (Jimenez-Mallebrera et al. 2005). Laminin-332 (laminin-5, kalinin, nicein, epiligrin) is widely distributed in the skin and is a component of anchoring filaments that connect the keratinocytes to the dermis (Rousselle et al. 1991). Laminin-511 (laminin-10) is among the most widely distributed laminins in tissues and is expressed abundantly for example in the skin (Määttä et al. 2001; Pouliot et al. 2002).

Several cellular receptors are known to recognize laminins. Laminin-111 is recognized by at least eight different integrins (Mercurio 1995). In addition to integrins, syndecans and α-dystroglycan also function in laminin binding. In fact, cell adherence is so crucial to laminin that it is nearly impossible to find

cell lines not expressing any receptors for it. The major laminin receptor integrins are α3β1, α6β1, α6β4 and α7β1 but also collagen receptor integrins α1β1 and α2β1 can recognize it (Elices and Hemler 1989; Ignatius et al. 1990; Belkin and Stepp 2000).

The major integrin type laminin receptors recognize certain LG domains at the C-terminal end of the laminin α chain. For collagen binding integrins α1β1 and α2β1 possible binding sites have been found in the N-terminal domain of the laminin α chain (Pfaff et al. 1994; Colognato-Pyke et al. 1995; Colognato et al. 1997; Ettner et al. 1998). In addition, using synthetic peptides, recognition sites for α2β1 have been predicted to reside close to the major laminin receptor integrin binding sites at the C-terminal LG modules (Underwood et al. 1995; Nomizu et al. 1997; Yokoyama et al. 2005).

2.4 Collagen receptor integrin αI domain evolution

Integrins are conserved throughout all metazoan phyla, from the simplest, cnidarians and sponges, to man (Figure 5) (Burke 1999; Hughes 2001). As a matter of course the unity of a multicellular organism requires the adhesion of cells to each other and to the ECM as well as communication between the cells. Integrins are the major group of molecules attaching cells to the ECM and relaying signals bidirectionally (Hynes 1992). BM components including laminin are highly conserved as is the ability of cells to adhere to these structures and form multilayered organisms. It has been speculated that two types of integrins evolved on early metazoans; a tripeptide RGD motif recognizing integrin and a laminin receptor integrin (Hynes & Zhao 2000).

Characteristic to collagen receptor integrins, that until now have been known from vertebrates only, is the insertion of an I domain in their α subunits. A similar domain (often called A domain) is found from many different proteins in Eukaryota. It's been hypothesized that the proteins containing the domain are involved in protein-protein interactions. The best known examples of vertebrate molecules containing A domains, in addition to the collagen receptor and leukocyte integrins, are the von Willebrand factor (vWF), and some ECM proteins, such as some collagens and matrilins (Colombatti et al. 1993; Tuckwell 1999). Some of the vWFA domains are capable of binding collagen, but with a mechanism and a binding site that are distinct from vertebrate collagen receptors, because the domains do not contain a functional MIDAS (Colombatti et al. 1993; Bienkowska et al. 1997; Nishida et al. 2003).

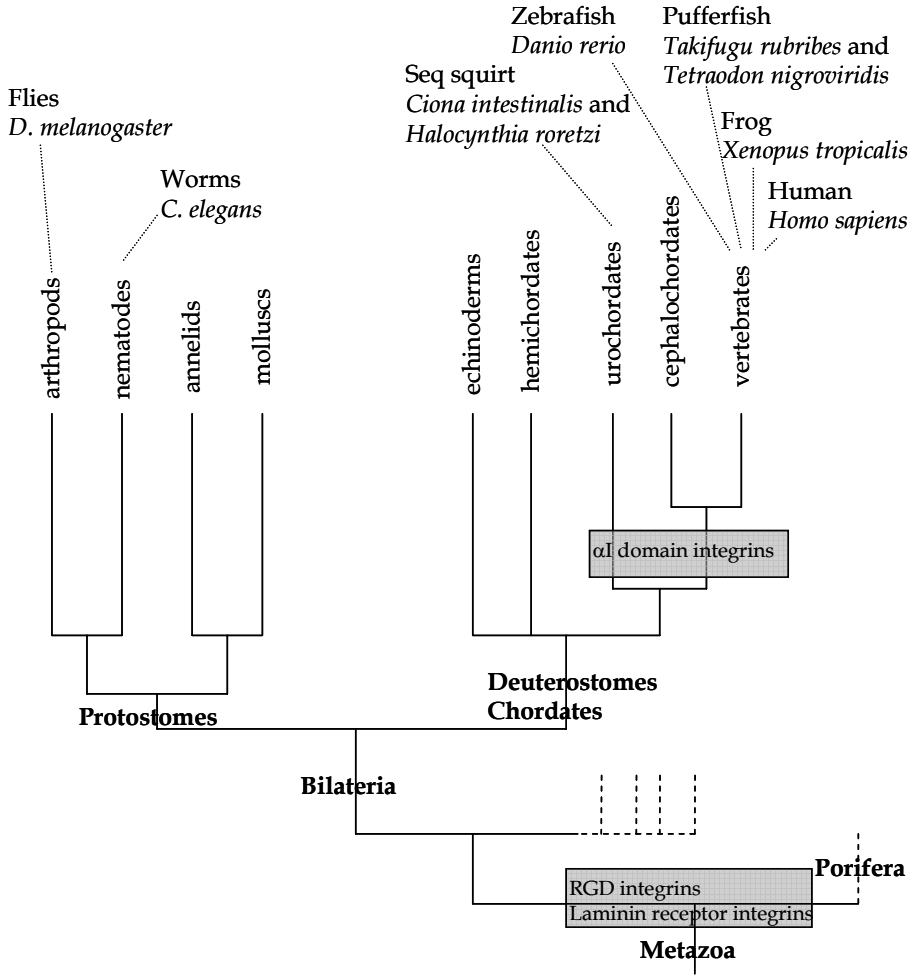

FIGURE 5 Evolutionary tree of bilaterian animals showing the distribution of integrins. Integrins are found throughout the metazoan phyla. Supposedly two types of integrins evolved in the early metazoa, which recognized either laminin or RGD. The examples of species are collected on the basis of their genome being sequenced, however the list is not extensive. Lengths of the branches are arbitrary. The figure is partly based on Dehal et al. 2002 and on Tree of Life Web Project http://tolweb.org/tree/ (20.12.2006).

All metazoan integrin β subunits have been predicted to have I domains, (βI, I-like domain) (Tuckwell 1999). In contrast, the insertion of the I domain into the α subunit seems to be a rather late event and confined to chordates only (Hynes & Zhao 2000; Miyazawa et al. 2001). Recently sequencing of the genome of *Ciona intestinalis,* an ascidian species of the most basal clade of chordates, revealed 11 integrin α subunits of which 8 contained an αI domain (Sasakura et al. 2003; Ewan et al. 2005). An αI domain containing integrin is known also from another ascidian species *Halocynthia roretzi* (Miyazawa et al. 2001).

The αI domain containing collagen receptor integrins are known from vertebrates only. It is hard to predict the functions of *Ciona intestinalis* and *Halocynthia roretzi* αI domains because these domains form a new separate phylogenetic group outside the vertebrate integrin αI domain groups (Huhtala et al. 2005). *Ciona intestinalis* αI domains contain a conserved MIDAS but lack the αC-helix, a characteristic of vertebrate collagen receptor integrins. However,

the αC-helix is not involved in binding of a collagenous peptide as aforementioned (Emsley et al. 2000).

3 AIM OF THE STUDY

The collagen receptor subfamily of integrins consists of four members; α1β1, α2β1, α10β1 and α11β1. They all bind to ligands with a special inserted or αI domain. The objective of this study was to characterize more carefully their ligand binding selectivity and how it is determined. As the integrins can be activated on the cellular level, the affects of activation on ligand binding selectivity were also studied.

Integrin αI domains and their binding to collagens seem to be rather late evolutionary events. However, sequencing of the early chordate *Ciona intestinalis* genome opened new avenues for research as integrins with αI domains were revealed. We investigated the possibility that these domains could function as collagen receptors.

The more detailed aims of the study where;

1) To produce the α10I domain as a recombinant protein and to prepare an integrin α10β1 expressing cell line and characterize the ligand binding selectivity

2) To characterize the structural features behind the ligand selectivities of the α1, α2 and α10I domains

3) To study the effect of integrin activation on ligand binding on the αI domain level

4) To study early integrin αI domains of the sea squirt *Ciona intestinalis* in order to gain insight into integrin αI domain evolution

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods are described in more detail in the articles and manuscripts I-IV.

4.1 Construction of expression vectors for human integrin αI domains (I-III)

The cDNAs encoding the human integrin α1I, α2I and α11I domains were synthesized with PCR as described earlier (Ivaska et al. 1999; Nykvist et al. 2000; Zhang et al. 2003). All constructs were checked by sequencing.

The integrin α10I domain cDNA was synthesized by RT-PCR (Gene Amp PCR kit PerkinElmer LifeSciences) from RNA isolated from KHOS-240 cells (human Caucasian osteosarcoma; ATCC). The α10I forward primer (5′-CAG GGA TCC CCA ACA TAC ATG GAT GTT GTC-3′) was designed to contain a *Bam*HI restriction site at the 5′ end. The reverse primer (5′- GGC TGA ATT CCC CTT CAA GGC CAA AAA TCC-3′) contained an *Eco*RI site at the 5′ end. The PCR, done in the presence of 2mM $MgCl_2$, consisted of forty cycles of denaturation at +94°C for 1 min, annealing at +67°C for 1 min and extension at +72°C for 2 min. The PCR product as well as the pGEX-2T (Amersham) and pMAL-c (New England Biolabs) vectors were digested with *Eco*RI and *Bam*HI restriction enzymes (Promega). The α10I domain cDNAs were ligated using either T4 ligase (Promega) or a SureClone ligation kit (Amersham Pharmacia Biotech) for the pMAL-c and pGEX-2T vectors, respectively. The DNA sequence was checked by DNA sequencing and by comparing it to the published sequence of integrin α10 (Camper et al. 1998). The plasmids were transformed into the *E. coli* BL21 strain for production.

Site-directed mutageneses of αI domain cDNAs were performed with the Quickchange™ system (Stratagene, La Jolla, CA, USA). The presence of mutations was ensured by DNA sequencing.

4.2 Assembly of the *Ciona intestinalis* integrin α1I domain coding gene (IV)

The *Ciona intestinalis* α1I domain (Acc. No. for the α1 subunit; ci010013118) was assembled from 30 overlapping oligonucleotides (Cybergene, Sweden) with PCR. Oligos were designed with DNABuilder (http://pga.swmed.edu/new_pga/Dreamweaver/dnabuilder/pga_DNABuilder.htm; 8.12.2006). The amplified DNA, purified from an agarose gel, was digested with *XhoI* and *EcoRI* restriction enzymes (Promega) and ligated into a similarly cut pGEX-4T-3 vector (Amersham Biosciences). The construct was checked by sequencing.

4.3 Recombinant αI domain production (I-IV)

Human integrin αI domains and the *Ciona intestinalis* α1I domain were produced as N-terminal glutathione S-transferase (GST) fusions in pGEX-2T (human α2I and α10I), pGEX-4T (human α1I and *Ciona intestinalis* α1I) and pGEX-KT (human α11I) vectors (Amersham Biosciences), or as a maltose-binding protein (MBP) fusion in the pMAL-c vector (human α10I) using *E. coli* BL21 cells. After the production the cells were harvested by centrifugation and stored at -70°C until purification. In the case of the MBP-protein fusion human α10I domain, the harvested cells were stored in the column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10mM β-mercaptoethanol, pH 7.4) at -20°C until purification.

GST-fusion proteins were purified using glutathione sepharose (Amersham). The cells were suspended in PBS buffer and lysed by sonication. Proteins were solubilized by incubation in 1% Triton-X100 for 30 min-1 h. All steps above were performed on ice. The solution was centrifuged and passed through a glutathione sepharose column. The column was washed with PBS, and the proteins were eluted with 30mM reduced glutathione.

For the MBP-fusion human α10I domain purification the cell suspension was thawed and sonicated on ice. The supernatant was incubated with amylose resin (New England Biolabs) overnight, washed with column buffer and eluted with 5mM maltose. The maltose was removed by allowing the fusion protein to bind to hydroxyapatite. The MBP-fusion protein was then eluted with sodium phosphate buffer.

The purity and folding of the recombinant fusion proteins were checked with SDS- and native polyacrylamide gel electrophoresis (PAGE) (Phast System, Amersham Biosciences) and the protein concentration was determined with the Bradford's method (Bradford 1976).

4.4 Matrix molecules used in the study (I-IV)

Matrix molecule	Supplier
collagen I, rat tail	Sigma
collagen I, human	Biomarket
collagen II, bovine	Chemicon International
collagen III, human	Chemicon International
collagen IV, Engelbreth-Holm-Swarm mouse sarcoma BM	Sigma
collagen IV, human	Chemicon International, Biomarket
collagen VI, human	Biodesign International, Biomarket
collagen V, human	Chemicon International
collagen IX, recombinant human	produced as described in Pihlajamaa et al. 1999
laminin-111, Engelbreth-Holm-Swarm mouse sarcoma BM	Sigma
laminin-211 (merosin), human	Chemicon International
laminin-332, rat	Chemicon International
laminin-511, human placenta	Chemicon International
tenascin, chicken	Chemicon International

4.5 Solid phase binding assays (I-IV)

Solid phase binding assays were performed on 96-well microtiter plates. The wells were coated with matrix proteins at 15-20μg/ml in PBS at +4°C overnight. The wells were then blocked with BSA containing Delfia Diluent II (PerkinElmer) for 1 h at RT. The same solution was used to detect the background binding. GST-fusion αI domains were diluted to suitable concentrations in Delfia Assay buffer (PerkinElmer) containing 2mM $MgCl_2$ or 10mM EDTA as indicated. Integrin αI domains were allowed to bind to the wells for 1 h, then the wells were washed three times with PBS containing $MgCl_2$. Signal was detected either with Europium-labeled anti-GST (PerkinElmer) (1:1000) or with anti-GST (1:8000) and Europium labeled protein G (1:100) that were diluted with Assay buffer (PerkinElmer) and incubated for 1 h at RT. The wells were washed three times, Delfia Enhancement solution (PerkinElmer) was added to wells and the signal was measured with a time-resolved fluorescence spectrophotometer (Victor2 multilabel counter, Wallac, or Envision™ 2100 multilabel reader, PerkinElmer).

4.6 Cloning of full length human integrin α10 (II)

Full-length integrin α10 cDNA (nucleotides 19-3525 of the published sequence, Camper et al. 1998; GenBank accession number AF074015) was prepared using RT-PCR (Promega) from RNA purified from human osteogenic sarcoma cells (SAOS-2 cells, ATCC). The primers introduced a *Bgl*II site into the 5′ end and a *Bam*HI site into the 3′ end. The digested cDNA was ligated into a similarly cut pcDNA3 expression vector (Invitrogen), and the sequence was verified by DNA sequencing.

4.7 Creation of human integrin α10 expressing CHO cell line (II)

The pcDNA3 vector containing the integrin α10 cDNA was transfected into chinese hamster ovary (CHO) cells (ATCC) using the FuGENE6 reagent (Roche Applied Science). Cell clones were isolated and selected with a neomycin analog G418 (400 μg/ml, Invitrogen) for three weeks. The clones were analyzed with RT-PCR (GeneAmp PCR, PerkinElmer) and immunoprecipitation for the expression of integrin α10.

For RT PCR, the total cellular RNA was purified (RNeasy minikit, Qiagen) and amplified (Gene Amp PCR kit, PerkinElmer) using sequence specific primers for α10. The PCR product was separated on a 1.5% agarose gel and stained with ethidium bromide.

For immunoprecipitation, the cellular proteins were metabolically labeled using 50 μCi/ml [^{35}S] methionine (Tran ^{35}S-label, ICN Biomedicals Inc., Irvine, CA, USA) for 18 h in methionine-free minimum essential medium (Sigma). The cells were washed, harvested and suspended in buffer containing 100mM n-octyl-β-D-glucopyranoside (Sigma) on ice. The cells were incubated in the buffer with occasional mixing by vortex for 15 min. The soluble fraction was precleared by incubating with protein A-sepharose (Amersham). Supernatants were immunoprecipitated with integrin antibodies (polyclonal β1 antisera, Heino et al. 1989; polyclonal α10 antisera was a kind gift from Dr. Evy Lundgren-Åkerlund, Lund, Sweden) for 12 h at +4°C. Immune complexes were harvested with protein A-sepharose (Amersham) and washed. The immunoprecipitates were analyzed on 6% SDS-PAGE gels under non-reducing conditions.

4.8 Cell spreading experiments (II)

Cell spreading experiments were performed essentially as described earlier (Nykvist et al. 2000). In short, microtiter plates were coated with matrix proteins

16.4 μg/ml and blocked with 0.1% BSA. Cells (10 000/well) in serum free media containing cycloheximide were allowed to spread on the matrix molecules for the indicated times. Non-adherent cells were removed by washing and the spread cells fixed. The numbers of total and spread cells were counted. The data are means ±S.D. of three parallel measurements.

5 REVIEW OF THE RESULTS

5.1 Production and purification of WT and mutant integrin αI domains (I-IV)

In order to study the binding of integrin αI domains to collagens, laminins and other matrix molecules, the αI domains were produced as recombinant proteins in an *E. coli* production system. The GST fusion expression constructs for human α1I and α2I domains already existed (Ivaska et al. 1999; Nykvist et al. 2000) and the construct for the α11I domain was prepared by others in cooperation with this study (Zhang et al. 2003). Since the human α10I domain had not been produced as a recombinant protein before, the MBP- and GST-fusion expression constructs for the domain were produced. The integrin α10I domain cDNA was prepared with RT PCR from RNA extracted from KHOS (human Caucasian osteosarcoma) cells (I). Synthetic oligos were used in the assembly of the *Ciona intestinalis* α1I domain gene. Recombinant αI domains were produced in an *E. coli* production system. Good yields of the purified recombinant human integrin α1I and α2I domains were obtained, (I-IV), whereas the production levels of the human α10I and α11I (I-III) as well as the Ciona α1I domain (IV) were constantly lower.

5.2 Collagen receptor integrin α10β1 and α10I domain binding to ligands (I and II)

The basic binding mechanism of human integrin αI domains has been shown to be cation dependent (Michishita et al. 1993). As this study was the first to characterize integrin α10I domain ligand binding, we were interested in whether the α10I domain would follow this rule. To study the effect of cations on α10I domain ligand binding, a metal ion chelating agent EDTA was used. EDTA was seen to inhibit binding of the α10I domain to collagen II (I; Fig. 1A),

which indicated a metal ion dependence for ligand binding similar to the other human integrin αI domains.

Various ECM molecules were tested with solid phase binding assays for α10I domain binding. The α10I domain was seen to mediate binding to fibril-forming collagen subtypes I, II, III and V, of which the binding to subtype V was clearly weaker than to subtypes I, II and III (I; Fig. 1B). The α10I domain recognized the BM molecule laminin-111 (laminin-1) whereas the ECM molecule tenascin did not support binding (I; Fig. 1C). When a more quantitative assay using a concentration series of the α10I domain was performed, the domain was seen to prefer network forming collagen type IV and beaded filament forming type VI over the fibril-forming collagen I (I; Fig. 3). The approximate K_d for both of these collagens was about 300-400nM (I; Fig. 3).

An integrin α10β1 expressing cell line was prepared in this study. CHO cells were selected because they do not express other collagen receptor integrins (Nykvist et al. 2000), but do express the integrin β1 subunit, a necessity for integrin heterodimer formation. Integrin α10β1 protein expression in a CHO cell clone was verified with RT-PCR and immunoprecipitation (II; Fig. 4A, B).

Cell spreading assays were performed to determine the ability of the α10β1 expressing cell line to spread on different collagens. The cell line was found to spread on fibril-forming collagens I-III and V, on network forming collagen IV, beaded-filament forming VI and FACIT collagen IX (II; Fig. 4C). The results of the cell spreading assays were in line with the α10I domain binding assay results. The binding was slightly better to collagen IV and VI that were favored by the α10I domain, even though the differences between different collagens were not that prominent on the cellular level.

5.3 Collagen receptor integrins as laminin receptors (III)

Collagen receptor integrins α1β1 and α2β1 are known to recognize laminins as well (Elices and Hemler 1989; Ignatius et al. 1990). Integrin α1β1 is frequently shown to act as a laminin receptor, but for α2β1 there has been some controversy in the results. Integrin α2β1 has been shown to work as a collagen receptor on some cells or both as a collagen and laminin receptor on others (Elices and Hemler 1989; Languino et al. 1989). The reason for this functional difference has been unknown. We wanted to better characterize the collagen receptor integrin αI domain's binding preferences for the laminin isoforms. Earlier, it had been shown that integrin α1β1 bound laminin-111 better than α2β1 (Kern et al. 1993).

Different laminin isoforms (laminin-111, -211, -332, and -511) were tested on solid phase binding assays for the integrin α1I, α2I, α10I and α11I domain binding. None of the domains recognized laminin-332, even though there are some earlier reports suggesting that integrins α1β1 and α2β1 might interact

with it (Orian-Rousseau et al. 1998; Decline and Rousselle 2001). Laminin-111 was found to be the best ligand for the integrin α1I domain (III; Fig. 1A, B, 6A). The approximate K_d determined for α1I domain laminin-111 binding was relatively good (122±24nM) which is comparable to the value for collagen I binding (100-200nM) (I; Fig. 2A). Laminins -211 and -511 mediated integrin α1I domain binding as well, the order of preference was mostly laminin-211 over -511. The laminin batches used here showed some variation.

The integrin α2I domain showed only weak binding to laminins -111, -211 and -511 (III; Fig. 1A, 2C, 4A). Occasionally no binding was detected to laminin-211, depending on the laminin batch. Either laminin -111 or -511 was preferred by the domain. As the binding to laminins was weak no approximations of the K_d were obtained.

The integrin α10I domain mediated binding to laminin -211 and -511 as well as to laminin-111 (I; Fig. 1C, III; Fig. 1C). The binding to the laminin-211 was slightly stronger than to the other laminin forms.

The integrin α11I domain recognized only laminin-111 in our assays (III; 1C). Binding was weak but comparable to that of the integrin α2I domain. No approximation of the K_d was obtained due to the weak interaction.

5.4 GFOGER independent binding (II)

GFOGER is the best characterized and highest affinity motif known for collagen receptor integrins. The crystal structure of the integrin α2I domain in complex with the hexapeptide reveals the structural details of the interaction (Emsley et al. 2000). FACIT collagen IX has no GFOGER sequence on any of its α chains. Still, as shown here, all collagen receptor integrin αI domains, α1I, α2I, α10I and α11I, mediate high affinity binding to collagen IX (III; Fig. 5).

5.5 Determination of the features affecting ligand binding selectivity in integrin αI domains (I- III)

Collagen receptor integrins are structurally very similar as shown by the crystal structures for the integrin α1I (Salminen et al. 1999) and α2I domains (Emsley et al. 1997). About half of the amino acid sequences of the integrin α1I and α2I domains is identical. It was known before this study that the integrin α1β1 and α2β1 binding specificities differed from each other with respect to their binding to collagens I and IV (Kern et al. 1993; Nykvist et al. 2000). In this study we wanted to determine the binding preferences of these domains to collagen VI as well. When characterizing the binding with solid phase binding assays it was discovered that these domains differed from each other in collagen VI binding. The integrin α1I domain showed significantly tighter binding than the α2I

domain (I; Fig. 2C, D). An approximate K_d for collagen VI was obtained for the α1I domain (≈200±80nM) (I; Fig. 2C).

Molecular modeling was used to identify sequence differences in close proximity to the ligand binding site MIDAS on αI domains that could account for the divergent binding specificities (I; Table I). One such amino acid identified was a positively charged R218 in α1I and α10I, whereas the corresponding residue was a negative D219 in α2I (I; Fig. 4). In the complex structure of the α2I domain the residue made contact with the collagen hexapeptide (Emsley et al. 2000). In order to test the effect of the residue on ligand binding selectivity, charge reversing swap mutations were created. In the α1I and α10I domains the arginine was mutated to aspartate and in the α2I domain the aspartate was mutated to arginine. For the α1I domain the R218D mutation changed the ligand binding pattern dramatically. Binding to collagens preferred by the wild type, collagens IV, VI (I; Fig. 5A) and IX (II; Fig. 8C) and laminin binding (III; Fig. 2B) weakened dramatically, but the binding to the fibril-forming collagen I remained the same (I; Fig. 5A). The corresponding mutation, R218D in the α10I domain, reduced binding to collagen IV, the collagen strongly bound by the wild type. However, as in the α1I domain mutant, the binding to collagen I was unchanged. (I; Table II). The opposite behavior was seen for integrin α2I D219R. The mutation decreased collagen I binding markedly, but not binding to collagen IV (I; Fig. 5B) or laminin (III; Fig. 2C). FACIT collagen IX binding was not affected by the mutation (II; Fig. 7D).

5.6 Integrin activation effects on ligand binding avidity and pattern (III)

The effect of integrin conformational activation on ligand binding avidity and pattern was studied to determine whether activation would account for the differences seen on the cellular level in integrin α2β1 ligand binding specificity (Elices and Hemler 1989). A gain-of-function mutation has been reported for the integrin α2I domain (E318W), where a salt bridge stabilizing the closed conformation is prevented by a mutation and thereby the open high-affinity conformer is favored (Aquilina et al. 2002). We identified the corresponding residue in the α1I domain and introduced a similar mutation into the domain (α1IE317W).

When testing the gain-of-function mutants with solid phase binding assays an increase in the avidity for the integrin α2I E318W domain towards collagen I was detected (III; Fig. 3B), the approximate K_d was about six times tighter (~4nM) than that of the wild-type (23nM). The ligand binding selectivity in contrast, was seen to decrease (III; Fig. 3A). The collagen binding levels that differed clearly from each other for the wild type α2I were equal for the mutant α2I. For the α1I domain, as well, the constitutively-active mutation decreased the ligand binding selectivity. The relative binding to collagen IV decreased

when compared to collagen I binding, which is a clear indication of the decreasing binding selectivity (III; Fig. 5A, B).

For both of the gain-of-function mutant integrin αI domains, α1I E317W and α2I E318W, an enhancement in the binding to laminins was detected (III; 4A, 6A). The avidity of the mutant α2I domain for laminins -111 and -211 rose markedly when compared to the wild type and approximate K_ds were obtained: laminin-111 (57±12nM) and laminin-211 (121±24nM) (III; Fig. 4B).

5.7 *Ciona intestinalis* αI domains and collagen binding (IV)

Sequencing the genome of the urochordate *Ciona intestinalis* (Sasakura et al. 2003) revealed the presence of integrins with αI domains, a domain earlier thought to be present only in vertebrates. In vertebrates, integrins with αI domains form two groups; the collagen receptor integrins and the leukocyte integrins. According to our phylogenetic analyses these ascidian integrins did not segregated with either of these groups (IV; Supplementary figure). According to the *Ciona* αI domain sequences, most of the MIDAS residues were conserved and molecular modeling suggested that a functional MIDAS is formed (IV; Fig 1B). As the phylogenetic analyses provided no information about the function of these integrins, we decided to produce one *Ciona intestinalis* αI domain as a recombinant protein to study it's function.

The *Ciona intestinalis* integrin α1I domain did not recognize GFOGER or any other related human integrin α2I domain recognition motif (IV; Fig. 3A). The *Ciona* α1I domain bound only to collagen IX of all the tested collagens (I, II, III, IV, and V), and it did not bind to laminin-111 (IV; Fig. 4A, B). The measured avidity for collagen IX (approximate K_d ≈300±70nM) (IV; Fig. 4B) was comparable to the values obtained for human αI domains for collagens. When the Mg-dependence of the ligand binding was tested by using the metal chelating agent EDTA and by mutating one of the metal coordinating residues, a threonine, to alanine (T99A), and thereby destroying the MIDAS, the binding was discovered to be metal-independent, unlike any other integrin αI domain so far (IV; Fig. 5).

6 DISCUSSION

Integrins are the major class of cell surface receptors mediating collagen binding. Another minor group is the tyrosine kinases called discoidin domain receptors 1 and 2 (DDRs) (Vogel et al. 2006) and the platelet glycoprotein VI (GPVI) (Moroi and Jung 2004). In the group of collagen receptor integrins, α1β1 and α2β1 are considered the primary collagen binding integrins with the widest tissue distribution. The integrin α10β1 and α11β1 expression profiles are more restricted. However, as shown here, in humans all the collagen receptors may be expressed concomitantly on some cell types such as chondrocytes (II; Fig. 2).

The collagen receptor integrin's major ligand binding domain is the αI domain. The specificity of the collagen receptor integrins α1β1 and α2β1 has been shown to reside on the αI domain (Kern and Marcantonio 1998). We studied the ligand binding of collagen receptor integrin αI domains at the molecular level. We especially wanted to investigate the ligand binding specificities of the α1I, α2I, and α10I domains and the factors governing these specificities. Laminin binding with all four collagen binding integrin αI domains (α1I, α2I, α10I and α11I) was also compared in parallel. The effect of integrin activation on ligand binding pattern and avidity was studied by mutating the α1I and α2I domains to produce constitutively active domains. Insight into integrin αI domain and collagen binding evolution was obtained by studying a αI domain containing integrin from a very basal chordate species *Ciona intestinalis*.

6.1 Collagen receptor integrin α1I, α2I and α10I domain and full length integrin α10β1 ligand binding (I-II)

The integrin α10β1 was characterized in 1998 and the integrin α11β1 only a year later (Camper et al. 1998; Lehnert et al. 1999). These are and will remain the latest additions to the integrin family of cell surface receptors. Since the ligand pattern for integrin α10β1 was not known and the α10I domain had not been

produced before as a recombinant protein, one of the aims here was to characterize the collagen receptor integrin α10β1 and its αI domain ligand binding more thoroughly and to compare it to other collagen receptor integrins. For that purpose an α10β1 expressing cell line was generated and α10I was produced as a recombinant protein. The cell spreading assay was selected as a method to study integrin α10β1 collagen interactions due to the better sensitivity of the assay over cell adhesion assays (Nykvist 2004). The recombinant integrin α10I domain ligand pattern was discovered to resemble that of the integrin α1I domain. Both domains showed good avidity for network forming collagen IV and beaded filament collagen VI. Meanwhile the avidity of the integrin α2I domain for collagen VI was relatively weak. The α2I domain clearly favored fibril-forming collagens over collagens IV and VI. In cell spreading assays, the full integrin α10β1 heterodimer as well, preferred non-fibrillar collagens IV and VI over fibril-forming ones, although the differences at the cellular level were not as high as seen in α10I domain assays. This very likely indicates that the solid phase assays used to study integrin αI domain binding are more sensitive than the cell spreading assays. The integrin α1β1 has been recognized as the main beaded filament forming collagen VI receptor on chondrocytes (Loeser et al. 2000). The discovery that integrin α10β1 also binds beaded filament collagen VI makes it yet another receptor for collagen VI in chondrocytes.

As the results here and elsewhere (Zhang et al. 2003) show that the integrin αI domain pairs α1I and α10I and α2I and α11I have similar ligand profiles, one might think that within the pair the receptors might compensate for each other better than a receptor from the other pair. However, the integrin pair α1β1 and α2β1 is often found co-expressed on the same cell type although the ligand profiles are different. Signaling mediated by the receptors α10β1 and α11β1 is not as yet known, which makes it difficult to predict their functional compensatory potential. Studies in which the integrin pairs α1β1 and α10β1 or α2β1 and α11β1 would be knocked out simultaneously could provide interesting insight into this question.

6.2 Collagen receptor integrin laminin binding (III)

Integrin αI domain binding preferences differed for different laminin subtypes. The integrin α1I domain showed generally the best avidity for laminins and favored laminin-111 over the other subtypes. When combining this information with the finding that integrin α1β1 prefers collagen IV, one can draw the conclusion that of the collagen receptor integrins, α1β1 is the best suited for BM binding. The integrin α2I domain preferred either laminin-111 or -511 over laminin-211, and the integrin α10I domain recognized laminins -211 and -511 as well as laminin-111. As the integrin α11I domain was shown to recognize laminin (laminin-111) as well as the other collagen receptor integrins, this

finding shows that all collagen receptor integrins are capable of binding to BMs to some extent if only with lower avidity than to collagen.

6.3 Metal ion-dependent binding mechanism of integrin αI domains (I)

All vertebrate collagen receptor integrins investigated so far have shown a metal ion-dependent binding mechanism. The sole ligand binding site in these receptors is the MIDAS situated on a groove on the top face of the αI domain. Not surprisingly, when the metal dependence of the α10I domain was studied, the ligand binding turned out to be metal dependent and involved MIDAS, like the vertebrate αI domains. The shallowness of the binding site needed to accommodate large fibrous ECM molecules like collagen raises the question of how the interactions formed in structures of this kind withstand collisions with free water molecules. Deeper binding sites would naturally provide better sheltering for the critical interactions. Molecular dynamics simulations with the αVβ3 βI domain MIDAS binding to an RGD motif have suggested a general strategy for how the metal ion stabilizes ligand binding in the I domains (Craig et al. 2004). According to that study it could be that one single water molecule is coordinated to the MIDAS metal ion in such a way that it prevents free water molecules from attacking the critical bonds. This method could possibly be utilized by other MIDA-sites as well, such as those in collagen receptor integrins.

6.4 Determination of the binding selectivity of integrin α1I, α2I and α11I domains (I-III)

We studied the molecular basis of the selective collagen binding in the collagen receptor integrin αI domains and by molecular modeling identified some potential amino acids contributing to the behavior. Residues α1IR218, α2ID219 and α10IR218, which occupied corresponding positions in each domain but whose charge varied, could possibly alter binding specificities. Site-directed mutagenesis utilized to reverse the charge proved our assumptions of the role of these residues in influencing ligand preference correct. The charge reversing mutation α1I R218D weakened binding to collagens IV, VI and IX and to laminins -111, -211 and -511, indicating that the residue R218 most probably is involved in interactions with these ligands. Collagen I binding, in contrast, was not affected. The mutation α2I D219R weakened collagen I binding, but it did not affect the binding to the collagens IV and IX nor to the laminins -111 and -511. Thus our results do not demonstrate a role for α2ID219 in the binding of

ligands other than collagen I. The binding of the mutant α2I D219R to laminin-211 was slightly enhanced, suggesting that the mutation created the domain more favorable for laminin-211 interactions. The mutation α10I R218D affected collagen IV binding most notably, indicating a possible direct role of the residue in binding. Thereby our results indicate that the charged residue at position 219 in the α2I domain and the corresponding position on the other collagen binding αI domains contributes to the characteristic binding profile of each receptor. Charge reversing mutations impaired the binding of the ligands preferred by the domain. Regardless of these findings, it is clear that a single amino acid alone can account for all the differences seen in the binding profiles. There must be other residues not yet characterized that affect the binding behavior as well. The integrin α2I domain has been crystallized in complex with the collagenous peptide GFOGER, showing a direct interaction of residue D219 among others (Emsley et al. 2000). Co-crystals of other ligand recognition motifs found from collagen I or other collagen types, like collagen IV do not exist. Neither are these available for other collagen receptor αI domains. Co-crystals could, however, reveal some new binding mechanisms utilized in the binding of various ligands.

6.5 Collagen IX binding by collagen receptor integrin αI domains and collagen receptor integrin binding motifs (II)

GFOGER is the best characterized high affinity binding motif in collagens that is recognized by the α1I, α2I and α11I domains (Emsley et al. 2000; Knight et al. 2000; Zhang et al. 2003). Even though not shown directly, the GFOGER sequence probably functions as a binding motif for the α10I domain as well. However, all the collagens do not contain the sequence. The FACIT collagen IX, which decorates the surfaces of collagen fibers, is one example, others being collagen III (Kim et al. 2005) and XIII (Zhang et al. 2003). All collagen binding integrins were shown to bind to collagen IX with high affinity. Thus it is obvious that the mechanism must differ from the well-described GFOGER based binding mechanism.

6.6 The effect of activation on integrin α1I and α2I domain ligand binding (III)

Characteristic for integrins is the relatively low affinity for ligands. Still they have to build mechanically enduring adhesions, as the function of integrins is to couple the contractile cytoskeleton to the ECM and to convey mechanical forces. It has been thought that the teamwork of integrins *in vivo,* summation of the

multiple weak interactions and the formation of focal adhesions account for the adequate binding strength.

To study the effect of activation on integrin ligand binding avidity and pattern we generated the mutants α1I E317W and α2I E318W that shifted the conformational equilibrium between the closed and open states towards the high affinity open state. The increase in the affinity of α2I E318W for collagen I was 5-10 fold when compared to the wild type. For leukocyte integrin αLI domain binding to intercellular adhesion molecule 1 (ICAM-1) an incredible 10 000 fold rise in the affinity was detected by the domain opening (Lu et al. 2001; Shimaoka et al. 2001). The relatively small increase in the affinity of α2β1 may reveal some fundamental difference between the leukocyte integrin and collagen receptor integrin function. The activity modulation may play a more important role for leukocyte integrins than for collagen receptors and therefore a bigger change in the affinity is obtained through allosteric modulation of the leukocyte integrin receptor.

The constitutively active αI domains, α1I E317W and α2I E318W, showed altered collagen selectivity suggesting that major functional differences exist between the two conformational states of the αI domain. Activation seems to reduce the differences seen for integrin α2I domain binding to various collagens and thereby reduces the selectivity of the receptor. The decreased selectivity for collagen was detected for the integrin α1I E317W domain as well.

For α2I E318W, the binding to laminins -211 and -511, in addition to laminin-111 as reported by Aquilina et al. 2002, was enhanced remarkably. For the wild type α2I the binding to laminins was so weak that no estimates for the K_d were obtained. Thus, our results proffer an explanation for the several observations of integrin α2β1 being capable of laminin binding only on some cell types (Elices and Hemler 1989; Chan and Hemler 1993) by showing that activation is prerequisite for α2I domain binding to laminin. Apparently, the conformational state of the integrin and its αI domain differs in various cell types, and in cells capable of binding to laminin, integrin α2β1 exists largely in the open state. That is to say that there seems to be differences in the activation state of integrins in different cells. The activation state of integrin could differ in different cellular domains as well. It would make sense if in migrating cells the integrins at the leading edge were active and those at the trailing end inactive.

Integrin adhesion to ECM molecules has been divided into two phases, the initial attachment and the following strengthening phase (Lotz et al. 1989). In light of these results showing that integrin ligand selectivity decreases when the receptor is activated one may speculate that for the primary recognition of ligands the selectivity may be more important (receptor not activated, higher selectivity) than for the later steps where strengthening of the contacts is obtained through binding of a larger selection of ligands and motifs (receptor activated by inside out mechanism, less selective binding). Probably for firm adhesion integrins need to be activated and maintain a high affinity conformation.

6.7 Evolution of integrin αI domain and collagen recognition (IV)

The sequencing of the genome of *Ciona intestinalis* and the revelation of αI domain containing integrins in this simple chordate species opened tempting prospects for collagen receptor researchers to study the evolution of integrin αI domains (Sasakura et al. 2003). We produced one of the αI domains (*Ciona intestinalis* α1I) as a recombinant protein to study the ligand binding.

It has been speculated that two types of integrins evolved in the course of evolution; the RGD recognizing and the laminin binding integrins (Hynes & Zhao 2000). Collagen receptor integrins have been assumed to have evolved significantly later. In our assays the *Ciona intestinalis* integrin α1I domain recognized neither laminin nor RGD containing fibrinogen (results not shown) but bound to collagen IX suggesting that when chordates radiated new types of integrins evolved with an ability to bind collagens.

The *Ciona intestinalis* integrin α1I domain recognized FACIT collagen IX with relatively good avidity. However, the ligand binding mechanism was metal-ion independent. Metal-independence suggests that the ligand binding site might be outside MIDAS. Regardless of how exotic it may sound, MIDAS independent collagen binding has been reported on other receptors too. The integrin αI domain homolog, the vWFA domain, binds collagen with a distinct mechanism. The vWFA domain lacks a functional MIDAS and the ligand binding site has been mapped to one of the sides of the domain in contrast to the MIDAS containing top face in collagen receptor integrin αI domains (Nishida et al. 2003).

The apparent GFOGER independent binding mechanism of the *Ciona* α1I domain and the fact that GFOGER sequences are absent in *Ciona* collagens indicates that MIDAS recognition of GFOGER evolved later, perhaps not until the evolution of vertebrates. The fact that the collagen IX binding site probably lies outside MIDAS in the *Ciona* α1I domain is biologically confusing. Ligand binding, when it takes place through MIDAS involvement probably always triggers signal transduction (outside-in signaling) in vertebrates by inducing conformational changes in the receptor. In theory, if ligand binding takes place without the MIDAS, and thus apparently without the possibility of a consequent conformational change propagating through the receptor, as in the case of *Ciona intestinalis* α1I domain binding, the binding process is independent of the cation concentration in the surroundings and no signals are necessarily transmitted. Whether this happens with a particular ligand only, is not known. For a cell this binding mechanism could mean that there are two categories of ligands; those that bind through MIDAS and affect cell behavior by triggering signal transduction, and those supposedly rarer ligands that bind but do not transmit signals but rather merely link the cell to the surroundings. However, the *Ciona intestinalis* integrin α1I domain contains the conserved glutamate residue that in vertebrates is used as the "intrinsic ligand" that binds to βI MIDAS and is critical in αI domain activation. The receptor seems to be

able to use the same activation mechanism as vertebrate integrins and thus to transmit inside-out signaling. Possibly the mechanism is used with ligands other than collagen IX.

7 CONCLUSIONS

In this study the collagen receptor integrin αI domains were produced as recombinant proteins in order to study the ligand binding selectivity and its regulation by mutating some of the assumed key residues. In addition, the effect of activation on the ligand binding pattern was studied. An integrin α10β1 expressing cell line was produced to study the function of the receptor. *Ciona intestinalis* αI domains were studied in order to gain insight into collagen receptor evolution.

The main conclusions of this thesis are;

1) The ligand binding profile of the integrin α10I domain, produced as a recombinant protein, resembles that of the integrin α1I domain. Both αI domains prefer non-fibrillar collagen types IV and VI over fibril-forming ones. The same result was attained with a α10β1 expressing CHO cell line.

2) Ligand selectivity of integrin α1I, α2I, α10I and α11I domains varies for collagens and laminins. Each αI domain has a characteristic ligand binding profile. Selectivity is at least partially determined by the residues α1IR218, α2ID219 and α10IR218 in the corresponding domains.

3) Integrin activation decreases the collagen binding selectivity for integrin α1I and α2I domains. Activation is prerequisite for integrin α2I domain laminin binding.

4) Early chordates may have had collagen binding integrins. The GFOGER dependent binding mechanism may, however, be a later invention.

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YHTEENVETO (Résumé in Finnish)

Kollageenireseptori-integriiniien evoluutio, ligandin sitomisvalikoivuus ja aktivaation vaikutus

Integriinit ovat suuri solureseptoriperhe, jonka jäsenet toimivat solujen kiinnittämisessä ympäristöönsä ja soluväliaineeseen. Integriinireseptorit muodostuvat kahdesta alayksiköstä; α ja β, jotka ovat ei-kovalenttisin sidoksin kiinni toisissaan muodostaen heterodimeerin. Integriinit ovat tärkeitä solujen normaalissa toiminnassa, mutta ne ovat osallisina myös patologisissa tiloissa kuten syöväs-sä, tulehduksissa ja verisuonitukosten synnyssä. Integriinien toiminnan perinpohjainen tuntemus on edellytys lääkkeiden kehittelyssä näiden sairauksien hoitoon.

Kollageeni on selkärankaisten yleisin soluväliaineen molekyyli. Kollageenia tunnistavia integriinireseptoreita tunnetaan neljä erilaista; α1β1, α2β1, α10β1 ja α11β1. Näille reseptoreille on yhteistä erityisen ligandia sitovan I-domeenin olemassaolo α alayksikössä. Ligandinsitomisen on havaittu olevan metallista riippuvaista ja tapahtuvan erityisen metalli-ionista riippuvan sitoutumiskohdan MIDAksen välityksellä. Kollageenireseptorit voivat sitoa samoja kollageenityyppejä, mutta erilaisilla voimakkuuksilla. Kaikkia neljää kollageenireseptoria voidaan ilmentää samanaikaisesti esimerkiksi rustosolussa.

Tässä työssä on selvitetty muun muassa kollageenireseptori-integriinien ligandinsitomisvalikoivuutta. Sen havaittiin vaihtelevan ja kullakin reseptorilla havaittiin olevan omanlaisensa ligandinsitomisprofiili. Integriinien α1I– ja α10I-domeenien ligandinsitomisprofiilit muistuttavat toisiaan, kuten myös intergiinien α2I– ja α11I–domeenien. Integriinit α1I ja α10I sitovat paremmin eisäikeisiä kollageenityppejä kuten tyvikalvojen kollageeni IV:ää ja helminauhasäikeitä muodostavaa kollageeni VI:a. Integriinit α2I ja α11I taasen sitovat säikeistä kollageeni tyyppiä I paremmin. Kollageenireseptori-integriinien havaittiin eroavan myös tyvikalvon laminiinin sitomisessa. Kaikki kollageenireseptorit sitoivat myös laminiinia, mutta heikommalla aviditeetilla kuin kollageenia. Kollageenireseptori-integriineistä α1β1:n havaittiin olevan parhaiten soveltuva tyvikalvojen sitomiseen.

Työssä pyrittiin selvittämään mitkä rakenteelliset tekijät αI-domeeneissa saavat valikoivan ligandin sitomisen aikaan. Integriinien αI domeenin ligandinsitomispinnalta, joka on hyvin samankaltainen kaikissa kollageenireseptoreissa, löydettiin yksi aminohappo joka vaikuttaa asiaan. α1I- ja α10I-domeeneissa kyseinen aminohappo on positiivisesti varautunut ja α2I- domeenissa samassa paikassa on negatiivisesti varautunut aminohappo.

Integriinin α2β1 on havaittu olevan kollageenireseptori joissakin soluissa ja sekä kollageeni- että laminiinireseptori eräissä toisissa soluissa. Syytä tähän käyttäytymiseen ei ole kovin hyvin tunnettu. Integriiniheterodimeerien on havaittu olevan erilaisissa konformaatioissa joilla on erilainen ligandin sitomisvoimakkuus. Integriinien αI-domeeni esiintyy myös ainakin kahdessa erilaises-

sa konformaatiossa, ”avoimessa” ja ”suljetussa”. Avoin muoto on korkean affiniteetin omaava muoto ja suljettu muoto vastaavasti matalan affiniteetin muoto. Tässä työssä tutkittiin integriinin αI-domeenin aktivaation vaikutusta ligandinsitomisvalikoivuuteen. Integriini α2I–domeenin aktivaation havaittiin olevan edellytys laminiinin sitomiselle. Kollageenin sitomisvalikoivuuteen aktivoituminen kuitenkin vaikutti heikentävästi. Oletettavasti alkuvaiheessa solun tunnistaessa ympäristönsä molekyylejä integriinien suurempi valikoivuus ligandin suhteen on tärkeää. Kun ympäritö havaitaan sopivaksi aktivoituneet integriinit voivat sitten tunnistaa laajempaa valikoimaa molekyylejä ja tunnistussekvenssejä niissä ja saavuttaa sitä kautta lujemmat adheesiokohdat.

Kollageenireseptori-integriinejä tunnetaan vain selkärankaisilta. Melko tuoreessa genomisekvensoinnissa on kuitenkin havaittu että myös alkeellisella selkäjänteisellä merituppeihin kuuluvalla *Ciona intestinali*ksella on myös αI-domeenillisia integriinejä. Tässä työssä tutkittiin yhden *Ciona intestinalis*-integriinin αI-domeenin ligandin sitomista. Sen havaittiin tunnistavan kollageeni IX:n, mutta selkärankaisista poikkeavalla metallista riippumattomalla mekanismilla. *Ciona intestinali*ksen genomista ei löytynyt GFOGER sekvenssin sisältäviä kollageeneja, eikä *Ciona intestinali*ksen α1I-domeenin havaittu GFOGER tai muita samantyyppisiä sekvenssejä tunnistavankaan. Havainto antaa viitteitä siitä, että kollageenireseptorit olisivat kehittyneet jo aiemmin kuin tähän asti on luultu. Selkärankaisten kollageenireseptorien GFOGER-riippuvainen kollageenin sitomismekanismi näyttää kuitenkin vaatineen kollageenien ja kollageenireseptori-integriinien samanaikaista evoluutiota.

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