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Petri Nykvist

Integrins as Cellular Receptors for Fibril-forming and Transmembrane Collagens

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ABSTRACT

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The two integrin-type collagen receptors α1β1 and α2β1 integrins are structurally very similar. However, cells can concomitantly express both receptors and it has been shown that these collagen receptor integrins have distinct signaling functions, and their binding to collagen may lead to opposite cellular responses.

In this study, fibrillar collagen types, I, II, III, and V, and network like structure forming collagen type IV tested were recognized by both integrins at least at the αI domain level. The αI domain recognition does not always lead for cell spreading behavior. In addition transmembrane collagen type XIII was studied. CHO-α1β1 cells could spread on recombinant human collagen type XIII, unlike CHO-α2β1 cells. This finding was supported by αI domain binding studies. The results indicate, that α1β1 and α2β1 integrins do have different ligand binding specificities and distinct collagen recognition mechanisms.

A common structural feature in the collagen binding αI domains is the presence of an extra helix, named helix αC. A αC helix deletion reduced affinity for collagen type I when compared to wild-type α2I domain, which indicated the importance of helix αC in collagen type I binding. Further, point mutations in amino acids Asp219, Asp259, Asp292 and Glu299 resulted in weakened affinity for collagen type I. Cells expressing double mutated α2Asp219/Asp292 integrin subunit showed remarkably slower spreading on collagen type I, while spreading on collagen type IV was not affected. The data indicated that α2I domain binds to collagen type I with a different mechanism than binding to collagen type IV.

In tissues collagen monomers form large fibrils immediately after they have been released from cells. The α2I domain binding data indicate that fibril formation affects the binding constant and at the same time reduces the number of putative binding sites available to the integrins. However, integrin α2β1 could still mediate spreading on fibrillar collagen and the contraction of floating collagen gels. The α1β1 cells could neither adequately spread on collagen fibrils nor mediate the contraction of collagen gels. These findings indicate that α2β1 integrin is a functional cellular receptor for collagen fibrils, while α1β1 integrin may only bind collagen monomers effectively.

Type XVII collagen, containing large collagenous domain called COL15, is a keratinocyte protein in hemidesmosomes or as soluable form in extracellular space. Assays showed that, unlike other collagens, COL15 was not recognized by the collagen receptors. Instead, the spreading on denatureted COL15 was mediated by α5β1 and αVβ1 integrins and inhibited by KGD-containing synthetic peptides. This suggests that the COL15 domain of collagen type XVII represents a specific collagenous structure, unable to interact with the cellular receptors for other collagens. After being shed from the cell surface it may support keratinocyte spreading and migration.

Key words: Collagen fibrils; collagen monomers; integrin-type collagen receptors; transmembrane collagens.

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This thesis is based on the following manuscripts or original publications, which are referred to in the text by their Roman numerals (I-IV). The proceedings have been reproduced with a kind permission of the American Society for Biochemistry and Molecular Biology Inc. as copyright holder. In addition, some unpublished, supplementary data from this project are presented.

I Nykvist, P., Tu, H., Ivaska, J., Käpylä, J., Pihlajaniemi, T. & Heino, J. (2000). Distinct recognition of collagen subtypes by α1β1 and α2β1 integrins. α1β1 mediates cell adhesion to type XIII collagen. J. Biol. Chem. 275: 8255-8261.

II Käpylä, J., Ivaska, J., Riikonen, R., Nykvist, P., Pentikäinen, O., Johnson, M., & Heino, J. (2000). Integrin α2I domain recognizes type I and type IV collagens by different mechanisms. J. Biol. Chem. 275: 3348-3354.

III Nykvist, P., Jokinen, J., Käpylä, J., Ivaska, J., Vehviläinen, P. & Heino, J. Cell adhesion to monomeric and fibrillar collagen (a manuscript).

IV Nykvist, P., Tasanen, K., Viitasalo, T., Käpylä, J., Jokinen, J., Bruckner-Tuderman, L. & Heino, J. (2001). The cell adhesion domain of type XVII collagen promotes integrin-mediated cell spreading by a novel mechanism. J. Biol. Chem. 276: 38673-38679.

ABBREVIATIONS

A	absorbance
aa	amino acid
ADAM	a disintegrin and metalloproteinase
αMEM	alpha Minimum Essential Media
ADMIDAS	additional metal ion-dependent adhesion site
A domain	I domain like structure in von Willebrandt factor
ATCC	American Type Culture Collection
BLAST®	Basic Local Alignment Search Tool
BP180	bullous pemphigoid autoantigen, collagen type XVII
cDNA	complementary deoxy ribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CHO	chinese hamster ovarian
CMD	carboxymethyl dextran
COL15	collagenous domain number 15 of collagen type XVII
CTGF	connective tissue growth factor
Cyr61	cysteine-rich 61 connective tissue growth factor (CCN1)
D	67 nm (234 aa) long repeat covering the overlap and gap region in collagen fibril
DDR	discoidin domain receptor
DEAE	diethylaminoethyl
DNA	deoxy ribonucleic acid
DMEM	Dulbecco´s Modified Eagle Medium
ECACC	European Collection of Cell Cultures
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetraacetic acid
EF	bivalent-cation-binding motif
EGF	epidermal growth factor
ERK	extracellular signal-related kinase
FACIT	fibril-associated collagens with interrupted triple-helices
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G-418	geneticin
GASEB	generalized atrophic benign epidermolysis bullosa
GST	glutationione S-transferase
GTP	guanosine 5´-triphosphate
GPIb	glycoprotein Ib alpha
GPV	glycoprotein V
Hepes	N-[2-hydroxyethyl]piperazine-N´-[2-ethanesulfonic]
ICAM	intercellular adhesion molecule
iC3b	complement 3 fragment
I domain	inserted, interactive domain
Ig	immunoglobulin
IPTG	isopropyl b-D-thiogalactopyranoside
LB	Lurian Broth
mAb	monoclonal antibody

MACIT	membrane-associated collagens with interrupted triple-helices, transmembrane collagens
MadCAM	mucosal addressin cell adhesion molecule
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MBP	maltose binding protein
MEK	MAP/ERK kinase
MES	2-[N-morpholino]ethanesulfonic acid
MIDAS	metal ion-dependent adhesion site
MOI	multiple of infection
MMP	matrix metalloproteinase
MW	molecular weight
NC	non-collagenous domain
NHS	N-hydroxysuccinimide
NIF	neutrophil inhibitory factor
NIH	National Institute of Health
NCBI	National Center for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PSI	plexin, semaphorin, integrin -type domain
RMS	root-mean-square deviation
RT-PCR	reverse transcription-polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
S.E.	standard error
TACE	tumor necrosis factor alpha converting enzyme
TGF	transforming growth factor
TIM	alpha/beta-barrel fold
Trp	tryptophane
uPAR	glycolipid-anchored urokinase receptor
UV	ultra violet
VCAM	vascular adhesion molecule
VLA	very late antigen
vWF	von Willebrand factor
vWFA	A domain of von Willebrand factor

1 INTRODUCTION

A major increase in the complexity of animal evolution at the transition from the unicellular protozoan to a multicellular metazoan occurred during the Cambrian explosion. The origin of these animals was accompanied by the appearance of extracellular matrix (ECM), which was used to provide mechanical and physiological support for eukaryotic cell unities. This ECM is a dynamic, network like aggregate, containing mainly collagenous glycoproteins, non-collagenous glycoproteins, and proteoglycans. Possibly one of the most important molecular inventions in the metozoan line was the development of collagens. Importantly, collagens are the most abundant proteins in a vertebrate body and particularly the subfamily of fibrillar collagens are essential for the formation of tissues characteristic for vertebras. It is known that the extracellular matrix has not only a structural role in tissues, but it also affects actively and regulates the attachment, proliferation, migration, differentiation, and metabolism of surrounded cells during phenomena such as development and morphogenesis.

The ECM related phenomena are mainly mediated by integrins and virtually all ECM glycoproteins are known to interact with integrins, which is common to all nucleated cells. Integrins are a large family of cell adhesion receptors, which can in addition have ECM interactions but also participate in cell-cell interferences that are largely confined to cells in the immune system. Together with matrix molecules and counter receptors they regulate among other development, the immune responses, hemostasis and tissue integrity. As well, integrin related pathological conditions like chronic inflammation and invasion and metastasis of cancer cells are known. At the moment 24 integrin αβ heterodimers have been found, formed by 18 different larger α subunits and 8 different smaller β subunits. Both subunits contain an extracellular domain, a transmembrane stretch and an intracellular domain with linkage to cellular signaling pathways. Subunits interact extracellularly with their NH_2-terminal parts to form a functional integrin dimer. Eighteen of these receptors recognize and bind matrix proteins. These include collagen binding integrins namely α1β1, α2β1, α10β1, and α11β1 dimers. Typical for collagen binding integrins are independently folding αI domains in the extracellular area, which has been shown to be responsible for ligand recognition in Mg^{2+} ion dependent way.

The role of collagens and collagen binding integrins and their

importance in cell-matrix interactions are reviewed in the following summary of literature. In this thesis both theoretical and experimental emphases are on classical collagen binding integrins, namely α1β1 and α2β1 dimers, fibrillar collagen types, and novel transmembrane collagen types XIII and XVII. The results section follows the order of original publications. In discussion section non-transmembrane collagens, collagen type I as fibrils and transmembrane collagens are dealt separately in their own unities without following the order of results. Also the methods in this study have been discussed shortly.

In publication I was investigated the ligand specificity of α1β1 and α2β1 integrins with recombinant αI domain in binding based studies and in spreading studies with α1 and α2 integrin subunit transfected cells. In publication II molecular bases of α2I domain based recognition of collagen type I by using site directed mutagenesis was studied. Also the role of αC helix in binding is discussed. In this study the binding and spreading data was further supplemented with molecular modeling and biosensor experiments. Manuscript III shed light on binding preference concerning monomeric and fibrillar type collagen I. The *in vitro* fibrillogenesis was carried out and binding/spreading experiments with different states of collagen were confirmed with immunoelectron microcopy and collagen gel contraction studies. In publications IV novel collagen type XVII was studied as well as binding/spreading studies supplemented with a lateral migration assay.

2 REVIEW OF LITERATURE

2.1 Family of collagens

At the moment the family of collagens consists of 27 closely related subtypes which are composed of α-chain peptides coded by 38 genes. In addition, 15 proteins that have collagen like domains have been found. Collagens are the most common group of structural components in vertebrates, which are present in all connective tissues, hence composing about one-third of body protein in man. The most abundant subtypes are found in the fibrils (for example types I, II and III in skin, tendons, cartilage and bone) or network-like structures (for example type IV in basement membranes) as supportive elements, while the less common collagen subtypes fulfill a great variety of, even unknown, more specialized biological functions (for example the soluble ectodomain of types XIII and XVII). Indicative of the biological importance of collagens, mutations within collagen genes lead to inherited diseases including osteogenesis imperfecta, chondrodysplasias, and some forms of osteoporosis and osteoarthritis (Prockop & Kivirikko 1995, and Myllyharju & Kivirikko 2001).

Collagens are defined to be partly triple helical components of extracellular matrix formed by three identical (homotrimer) or even three different (heterotrimers) α helix peptides. Characteristically, these peptides contain Gly-X-Y -repeats, i.e. helix forming collagenous motifs, in their amino acid sequence. The length and orientation of triple helical domains, the quality and quantity of nonhelical domains, the amino acid residues in X- and Y-positions and their degree of post-translational modification vary between the collagen subtypes. Due to these primary characters of monomers and the polymeric structures of monomer forms, collagens can be divided into two subgroups; namely uninterrupted triple-helix containing fibril-forming collagens (types I, II, III, V, XI, and XXVII), and the non-fibril-forming collagens (types IV, VI, VII, VIII, IX, X, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX, XXI, XXIII, and XXV). Collagen like proteins do contain Gly-X-Y -repeats and they do form triple-helical structures, but they are not defined as collagens since they are not structural components of the extracellular matrix (Myllyharju & Kivirikko 2001, Koch et al. 2001, Hashimoto et al. 2002, Banyard et al. 2003).

Each collagenous domain of α-helix is left-handed coiled into a type II

helical conformation and further three α-helices are twisted around each other into a unique, right-handed super-helix that repeats at slightly less than every third amino acid. Within the repeats, the glycine in every third position is necessary for a coiled structure allowing the dense packing of a super-helix core from which only the carbonyl oxygen remains accessible to form hydrogen bonds with the solvent. In the triple helical area the amino acids in X- and Y-positions are typically proline and lysine or their hydroxylated derivatives. These residues are important for the formation of hydrogen bonds and water bridges between the three helices, which improve the thermal stability and limit the rotation of N-C peptide bonds in the polypeptide chain. Importantly in addition to intramonomeric interactions, the hydrophobic and charged side chains of X- and Y-position amino acids are located on the surface of collagen monomer, which enable precisely ordered polymerization with self assembly into supramolecular aggregates when necessary, and characteristics for the subtype's essence (Viidic 1996, Adachi et al. 1997, and Myllyharju & Kivirikko 2001).

2.1.1 Fibrillar collagens

Fibril-forming collagen types I, II, III, V, XI and XXVII were originally known as group I collagens. They form the structural bases of most tissues and have the same characteristic size of monomers and the typical rope-like structure when self-assembled into the collagen fibrils. Typically, each monomer contains a large non-interrupted triple helical domain composed of 338 Gly-X-Y -repeats in each α-chain and in addition short terminal non-helical domains. In all fibrillar collagen subtypes, genes coding these repeats are considered to be ancestrally amplified, and consequently to have 44 genetically related exons the size of which being different multiples of 9 bp (=1 Gly-X-Y -repeat; Yamada et al. 1980, and Vuorio & Crombrugghe 1990). As a result of glycines and the interval of helical repeats, 3 left handed α-strands can wrap around each other to form a polypeptide monomer having the dimensions of 300 nm in length and 1.5 nm in diameter. In physiological conditions however, the lifetime of fibril forming collagens as single monomers is short. Collagen monomers assembly spontaneously to fibrils, which can be composed of either the same collagen subtype monomers or different subtype monomers. Based on this the fibrils formed are called either homotypic or heterotypic, respectively (Prockop & Kivirikko 1995). Typically the fibrils formed, when labeled with heavy metals, are periodically electron dense due to alternating molecular gaps and overlapping regions that therefore cross-striated (D periods) in electron microcopy. *In vivo*, the dimensions of fibrils formed, and further the degree of histological fibril organization are related to mechanical properties and vary depending on the physiological function to fulfill in tissues (Myllyharju & Kivirikko 2001 and Ottani et al. 2001). For more detailed description concerning collagen types I, II, III, and V see chapter 2.1.1.2.

2.1.1.1 Synthesis of fibril-forming collagens

Fibrillar collagen forming peptides are synthesized at membrane-bound ribosomes as larger precursors, called proα-chains or procollagen molecules (Mr 125-240 kDa). In addition to collagenous domains these precursors contain highly conserved non-collagenous C-propeptide (250 aa) and more variable N-

propeptide (50-500 aa) that are linked to the triple helix by short sequences, called telopeptides (Eyre et al. 1984, Dion & Myers 1987, Miller & Gay 1987, van der Rest & Garrano 1991, and Kielty et al. 1993). Furthermore, the N-terminal region of procollagen molecule contains a signal sequence (preprocollagen) which targets the proα-chain to the lumen of the endoplasmic reticulum where it undergoes extensive co-translational and post-translational modifications until the peptide chains fuse to form a triple helix.

After signal peptide cleavage an average of half the proline residues in Y-position are hydroxylated by prolyl-4-hydroxylase enzyme. Importantly, these hydroxyl groups increase the hydrogen bonding within and between the chains, which stabilize the mature collagen molecule at physiological temperatures (Kivirikko et al. 1992, Kielty et al. 1993, Kivirikko 1993, Bella et al. 1995, Kramer et al. 1998, Kivirikko & Pihlajaniemi 1998, and Lamande & Bateman 1999). Some prolines in X-position are further hydroxylated by prolyl 3-hydroxylase, but only with the presence of 4-hydroxyproline in the Y-position. The rate of 3-hydroxyproline varies between the collagen types, even within collagen type, and the biological significance of this hydroxylation is not known (Kivirikko & Myllylä 1982, Kielty et al. 1993, Kivirikko, 1993 Prockop & Kivirikko 1995, and Kivirikko & Pihlajaniemi 1998).

Lysyl hydroxylase hydroxylates some of lysines in Y-position to enable the attachment of O-linked carbohydrates, i.e. galactose to hydroxylysine and further glucose to galatoctosylhydroxylysine. In addition to a linker function, lysine hydroxylation may regulate the lateral packing of collagen molecules within fibrils and take part in the intra- and intermolecular cross-links providing tensile strength and mechanical stability of mature collagen (Kivirikko & Myllylä 1979, Kivirikko et al. 1992, Kivirikko 1995, and Kivirikko & Pihlajaniemi 1998). Some asparagines and serine residues are targets of N-glycosylation and glycosaminoglycan attachment, respectively (Kivirikko & Myllylä 1985, and Kielty et al. 1993). When processed in endoplasmic reticulum hydrophobic regions of procollagen peptides interact with molecular chaperons which prevent premature aggregation and assist later in the helix formation (Nakai et al. 1992, Gething & Sambrook 1992, Satoh et al. 1996, and Wilson et al. 1998).

Triple helix formation starts by folding of the C- and N-propeptides in each of the three proα–chains, which is followed by formation of intramolecular disulfide bonds within them (Bächinger et al. 1981, and Doege & Fessler 1986). The C-propeptides ensure the collagen type specific assembly of procollagen α-chains and creates the nucleation site via intermolecular disulfide bonds from which the triple helix formation proceeds in a C to N direction (Olsen et al. 1976, Engel & Prockop 1991 and McLaughlin & Bulleid 1998). Helix formation completes with the association of N-propeptides (Bächinger et al. 1980). Completely folded propeptides are transferred to the Golgi apparatus for further processing of N-glycosylations and for dissociation of molecular chaperons (Hammond & Helenius 1995, and Satoh et al. 1996). Procollagen molecules are condensed in granules that are secreted to the extracellular space by exocytosis (Bonfanti et al. 1998).

After secretion the propeptides are cleaved off by specific procollagen metalloproteinases leaving the part of telopeptides (11-26 aa) on the terminal regions of collagen molecule (Prockop & Hulmes 1994 and Prockop et al. 1998). However, in some cases, especially concerning collagen types I and III, the N-terminal propeptide is restored mainly in monomers situated on the surface of

developing fibril, which is considered to be part of the terminal proteinase activity based endogenous diameter control. In other words, the uncleaved N-propeptides are supposed to limit further accretion of collagen molecules onto growing fibrils (Hulmes 1983 and Fleischmajer et al. 1985). The C-terminal propeptide has control over the life time of collagen as a single monomer. After removal of this globular domain solubility of collagen monomers decreases and they start to form fibrils spontaneously assisted by the remaining telopeptide domains. For detailed mechanism of fibrillogenesis see chapter 2.1.1.3. In addition to regulatory function, terminal non-helical domains are primary sites for covalent intermolecular cross-linking when assembled to fibrils (Eyre et al. 1984 and Prockop & Hulmes 1994). For this purpose the lysine and hydroxylysine residues within telopeptides are oxidatively deaminated to corresponding aldehydes by copper dependent lysyl oxidase (Kagan & Trackman 1991, Kielty et al. 1993, and Prockop & Kivirikko 1995). These aldehyde groups are used spontaneously for covalent crosslinks between two aldehydes or aldehyde and lysine or aldehyde and lysine derivative to link the quarter staggered monomers together, which therefore gives the mechanical strength for fibrils (Kielty et al. 1993 Prockop & Kivirikko, 1995 and Kivirikko 1995).

2.1.1.2 Monomeric family members in fibrils

As indicated by Roman numeral collagen type I is the first collagen type determined in the family. It is the most abundant collagen subtype synthesized by fibroblasts or osteoblasts and therefore occurring in most tissues. Type I is prominently present in the fibrillar bundles of dermis, ligaments, tendons and organic matrix of bone. The most common type I molecule is a heterotrimer composed of two identical α1(I) chains (gene *COL1A1*; AF017178) and one α2(I) chain (gene *COL1A2*; AF004877). In addition, based on renaturation experiments and observations concerning embryonic tissues, dermis, and dentin, also less abundant type, namely homotrimer containing three α1(I) chains has been reported (Tkocz & Kühn 1969, Jimenez et al. 1977, and Wohllbe & Carmichael 1978). In some tumor and fetal tissues the existence of transheterotypic combination of two α1(I) chains with collagen type III forming α1(III) chain has been observed (Pucci-Minafra et al. 1993). In the α chains of type I monomers, the lysine residues are rarely hydroxylated (27 % of α1(I) chain and 38 % of α2(I) chain lysines) forming few sites for glycosylation (Eyre 1980 and Kivirikko et al. 1992). Type I monomers are usually located in thick bundles (Ø from 80 nm to ≥ 1000 nm) containing small amounts of other collagen type monomers, especially types III, V and XII, as the fibril core or surface (Myllyharju & Kivirikko 2001). In addition to other collagen types mentioned, type I interacts specifically with dozens of different collagen-associated molecules and cellular receptors. Molecular interactions mapped to specific and unique sites in collagen type I monomer include association sites for fibronectin, decorin core protein and dermatan sulfate side chains (Di Lullo et al. 2002). Importantly, the first binding sites for classical collagen adhesion receptors, namely α1β1 and α2β1 integrins, have been mapped based on studies of synthetic peptides with $GFP^{(OH)}GER$- and $GLP_{(}^{OH)}GER$-motifs ($P^{(OH)}$= hydroxyproline) in the collagenous regions of α(I) chain peptides (Knight et al. 1998, Knight et al. 2000, and Xu et al. 2000). Recently, also the α11β1 integrin has reported to recognize $GFP^{(OH)}GER$-motif (Zhang et al. 2003). Human α1(I)

chain contains along with a potential proline hydroxylation as a post-translational modification both $GFP^{(OH)}$- and $GLP^{(OH)}$-motifs in GER-context. Correspondingly, from the human α2(I) chain two GLPGER-motifs can be found. The existence of these amino acid sequences are highly conserved during collagen type I molecular evolution. According to BLAST® software (NCBI) and NCBI amino acid data base, both or either one of these motifs can be found in α(I) peptide chains of rat (α1(I): CGRT1S, α2(I): AF121217), mouse (α1(I): U08020, α2(I): BC007158), bovine (α1(I): CA11_BOVIN2), chicken (α1(I): P02457, α2(I): M25984), dog (α1(I): AF153062), sea urchin (*Strongylocentrotus purpuratus*; α2(I): M92040), frog (α1(I): AB015440), *Dario rerio* (α2(I): AJ318213), and *Cynops pyrrhogaster* (α1(I): AB015438) α(I) peptide chains. Both human α(I) chains have together 17 other than GFP- or GLP-related GER-motifs and their importance in integrin and collagen specific interaction is not known. The non-mapped molecular interactions include reported binding with DDR-1, DDR-2 syndecan-1, tenascin-C and von Willebrand factor. In general, the active sites for molecular interactions seems to concentrate to three key areas of chain peptides in collagen type I monomers. The functional domains include the region near the N-terminus covering residues 80-200, a middle area containing residues 680-830, and the terminal area starting from residue 920 continuing to the end of the C-terminus (Agarwal et al. 2002, and Di Lullo et al. 2002). Quantitative and qualitative defects in collagen type I based matrix interactions can be caused by hundreds of different mutations within *COL1A1* and *COL1A2* genes, which lead to severe connective tissue disorders. These inherited clinical phenotypes include osteogenesis imperfecta and certain types of osteoporosis and Ehlers-Danlos syndrome (Myllyharju & Kivirikko 2001, and Di Lullo et al. 2002). According to literature, collagen type I interaction as fibrils with collagen binding integrins is not shown.

Type II collagen molecule is purely homotrimer having three identical α1(II) chains (gene *COL2A1*; L10347; Bateman et al. 1996). As a major collagenous component of hyaline cartilage fibrils (from 80 to 95 % of total collagen volyme), type II co-exists covalently linked to the fibrillar collagen type XI and fibril-associated collagen type IX in molecular ratio of 8:1:1, respectively. In adult vertebras small amounts of collagen type II can be found in the vitreous body of the eye and the intervebral discs with same molecular context (Vaughan et al. 1988, Eyre 1991, Brewton & Mayne 1992 and Bateman et al. 1996). In addition, it can be observed also in some other non-chondrogenic tissues during development. Collagen type II is typically absent in type I containing tissues and its coexistence with collagen type V has been reported (Prockop & Kivirikko, 1995, Keene et al. 1995, and Fertala et al. 1996). Collagen type II forms intimate interactions with cartilage proteoglycans which seem to regulate the fibrillogenesis either by accelerating or retarding this process, respectively (Scott 1988 and Kuijer et al. 1988). At least decorin, lumican and fibromodulin has reported to participate to this process both *in vivo* and *in vitro* (Vogel et al. 1984, Ezura et al. 2000 and Säämänen et al. 2001). To regulate the specific assembly of type II molecules to relatively thin fibrils (Ø 20-200 nm), monomers have extensively hydroxylated lysine residues (47 % of all lysines) which are further extensively glycosylated if compared to collagen types I and III (Kivirikko & Myllylä 1979, Eyre 1980, Kivirikko et al. 1992, Fertala et al. 1996, and Notbohm et al. 1999). Also the kinetics of fibrillogenesis of collagen type II, and the orientation of monomer within fibrils (C-C termini of fibril) seems to differ from collagen type I (N-N termini of fibril) at least in experimentally

induced fibril assembly (Fertala et al. 1996). Kinetic differences include the considerably higher critical concentration of monomers in reaction to proceed, the longer lag time for intermediate formation, and the slower propagation of fibrils if compared to homotypic collagen type I based fibril assembly (Fertala et al. 1994). Similar to collagen type I α(I) chains, the human α1(II) chains contain one GFPGER- and one GLPGER-motif, i.e. potential recognition site for collagen binding integrins (Knight et al. 1998, Knight et al. 2000, Xu et al. 2000, and Zhang et al. 2003). Both or either one of these sequence groups have been reported for mouse (B41182), bovine (CA12-BOVIN1), pig (AF201724), and frog (AAA49678) of collagen type II a chain peptides. Dozens of mutations in the human COL2A1 gene have been reported including defects leading to several types of chondrodysplacias (Myllyharju & Kivirikko 2001). The role of integrin α1I domain in collagen type II - α1β1 integrin interaction has not been shown.

Collagen type III is a homotrimer composed of three α1(III) chains (gene *COL3A1*; P02461; Bateman et al. 1996). However, in some tissues, as described earlier, collagen type III α1 chain can also associate with two α1(I) chains therefore forming "a collagen type mixed" heterotrimer (Pucci-Minafra et al. 1993). Type III α chains peptides have very low content of hydroxylysine (17 % of all lysines), a low degree of glycosylation, and have heavily hydroxylated proline residues (54 % of all prolines; Eyre 1980 and Kivirikko et al. 1992). In addition, type III does differ from other mature fibrillar collagen molecules by having intermolecular disulphide bond between the α1(III) chain peptides at the C-terminal end of the triple-helical region (Epstein Jr & Munderloh 1975). This collagen type exists in most soft tissues containing type I therefore being the major collagen of blood vessels and abundantly found in dermis and intestine. Collagen type III is known to be important for organ development in growth and later for the maintenance of physiological function in adult tissues (Olsen 1995, and Bateman et al. 1996). Importantly, in blood vessels type III is the component of basement membrane of endothelial cells therefore interacting in blood vessel injury with homeostatic components and inducing thrombogenesis at least experimentally *ex vivo* (Sakariassen 1990). Fibrils formed by collagen type III monomers are thinner than type I fibrils, the structure being adapted to tissues exhibiting a high degree of elasticity (Keene et al. 1987, and Bornstein & Sage 1989). Further, type III monomers often retain permanently the N-terminal propeptide as part of endogenous fibril diameter control on the surface of fibrils therefore result in a product called type III pN-collagen (Fleischmajer et al. 1985). Interestingly in all sequences reported so far and with respect to collagen types I and II, the α1(III) chains do not contain any known potential binding sites for collagen binding integrins (Knight et al. 1998, Knight et al. 2000, Xu et al. 2000, and Zhang et al. 2003 in press). More than 100 mutations have been described in the *COL3A1* gene leading to connective tissue manifestations including type IV Ehlers-Danlos syndrome (for reference see Di Lullo et al. 2002). The role of integrin αI domains in collagen type III - α1β1/α2β1 integrin interaction has not been shown.

The most common collagen type V monomers, originally called AB type collagen, can be composed of three genetically distinct gene products, namely α1(V) (gene *COL5A1*; P20908), α2(V) (gene *COL5A2*; P05997) and α3(V) (gene *COL5A3*; XM009025) chains. Due to structure and size of gene *COL5A1* it is considered to be ancestrally diverged from more conserved collagen types I-III coding gene group (Takahara et al. 1991). In addition α1(V) chains lack some potential cross-linking sites in both telopeptides and it has an unutilized

glycosylation site in the N-terminal propeptide (Greenspan et al. 1991). Instead, type V is very much allied to collagen type XI. Interestingly, these different type α1 chains of collagen types V and XI can even functionally replace each other in cartilage (α1(XI) -> α1(V)) and bone matrix (α1(V) -> α1(XI)) to form transheterotypic monomers (Eyre et al. 1987, and Niyibizi & Eyre, 1989). For pure α(V) chain based homotrimeric a combination of three α1(V) chains and the existence heterotrimeric combinations of (α1(V))2α2(V) and α1(V)α2(V)α3(V) chains have been observed (Eyre, 1980, Eyre et al. 1987, and Bateman et al. 1996). Most of biochemical and functional data concerning collagen type V is from experiments carried out using the most abundant type of monomers with [α1(V)]2α2(V) chain combination, which is present in most tissues (Chanut-Delalande et al. 2001). Other chain combinations have meager existence in vertebrate organs. Heterotypic combination of α1(V)α2(V)α3(V) chains is merely found in the placenta (Niyibizi et al. 1984) and homotypic combination of α1(V) chains is likely to be present in embryonic tissues (Haralson et al. 1980, Kumamoto & Feeler 1980, and Moradi-Ameli et al. 1994). In addition to these well known α chains, existence of α4(V) chain was recently published. It is expressed abundantly by migrating and premyelinating Schwann cells together with the α1(V) and α2(V) chain (Chernousov et al. 2000). Collagen type V monomers coexists usually with collagen type I in heterotypic fibrils being probably involved in diameter control of these aggregates, in addition sharing number of structural similarities with cartilagenous fibrils formed by collagen types II, IX, and XI is seen (Birk et al. 1988, Mendler et al. 1989, Miller & Gay 1987 and Bateman et al. 1996). Like collagen type II, type V molecules have high content of hydroxylysine (depending on a chain from 57 to 74 % of all lysines) which are heavily glycosylated. In contrast collagen type V has relatively low content of alanine if compared to the other monomers of fibrillar collagens (Eyre 1980). Collagen type V has several molecular interactions. It interacts at least with DNA, heparin sulfate, thrombospondin, heparin and insulin (for references see access number P20908). Only chicken α1(V) chain (AF137273) has been reported to include potential $GFP^{(OH)}GER$ binding motif for integrins (Knight et al. 1998, Knight et al. 2000, Xu et al. 2000, and Zhang et al. 2003). Collagen type V displays exceptionally rare clinically manifesting mutations. However, recently reported single base changes in the α1(V) and α2(V) chains can disturb the collagen type V based matrix assembly therefore producing an Ehlers-Danlos syndrome type I and type II like symptoms (Michalickova et al. 1998, Richards et al. 1998, and Giunta & Steinmann 2000). The role of integrin α2I domain in collagen type V - α2β1 integrin interaction has not been shown.

2.1.1.3 Fibrillogenesis and fibrils formed - a collagenous puzzle

The following presentation is summarized in Fig. 1. The mechanism of collagen fibril formation *in vitro* has been under extensive research for decades, with the solubilized monomers of fibrillar collagens forming supramolecular aggregates in experimental conditions, which are at least apparently parallel to those fibrils found in tissues (Brodsky & Eikenberry 1982, and Brodsky et al. 1982). Most of this primary information about fibril assembly has been resulted in studies which have been carried out by reconstitution of collagen type I extracted with cold acidic solutions from tendons and skin. In these studies monomer polymerization to fibrils, phenomenon called fibrillogenesis, proceed when

collagen preparation is incubated at of 20 °C or over and in near physiological buffer conditions (Wood & Keech 1960, Wood 1960a, and Wood 1960b). Despite the general similarity of fibrils *in vivo* and *in vitro* some differences have been observed. Acid extracted collagen monomers do not usually form fibrils that have the same diameter than the fibrils from which they were extracted. Furthermore, fibrils formed are not as round and not as tightly packed as native ones. To exclude these differences caused by acidic extraction, the procollagen based *de novo* system to study fibril assembly was developed (Miyahara et al. 1984, Kadler et al. 1990a, Kadler et al. 1990b, and Romanic et al. 1992).

Based on observations made in both *in vitro* and *de novo* measures, the experimentally induced fibril formation is an entropy-driven reaction and has typical features of a crystallization process. The reaction has a critical concentration of monomers or oligomers to proceed and its value is inversely proportional to the polymer growth constant. The higher concentration of cross-linked collagen oligomers (dimers, trimers and higher compounds) are in the solution, the faster the rate of fibril assembly. The solution turbidity measurements indicate biphasic, sigmoidal reaction kinetics including the lag (initiation) phase, propagation (growth) phase and finally the equilibrium state. The length of the initiation phase is inversely related to the collagen concentration and it varies substantially from experiment to experiment. The turbidity change measured is approximately proportional to the amount and/or diameter of fibrils formed in the reaction (Wood & Keech 1960, Wood 1960a, Wood 1960b, Na et al. 1986b, Na et al. 1989, and Kadler et al. 1996 and Prockop & Fertala 1998a). According to indirect evidence it is probable that the initiation of assembly involves subtle changes in a small portion of the molecules forming "nucleation centers" analogous to the formation of crystals from inorganic salt solution driven by the negative free energy change. This energy change is due to loss of solvent molecules from the surface of monomers when the surface area/volume ratio in minimized in the polymerization (Wood & Keech 1960, Cassel et al. 1962, Frigon & Timasheff 1975, and Comper & Veis 1977). The fibril nuclei are suggested to exist as metastable intermediates which have a long life time at low temperatures indicated by a considerable shorter lag time after repeated heating and cooling cycles (Comper & Veis 1977). This phenomenon, called thermal memory, is lost after enzymatic removal of N- and C-terminal telopeptides. These axially contracted hair pin loop like structures seems to alter the kinetics of fibrillogenesis by lowering the activation energy for nucleus formation or stabilizing the nucleus formed against dissociation during the lag phase (Chandrakasan et al. 1976, Meek et la., 1979, Hulmes et al. 1977, Helseth et al. 1979, Helseth & Veis 1981, and Capaldi & Chapman 1982). However, the role of telopeptides as domains containing all the necessary information for monomer-monomer interaction is under discussion. Instead, the consensus view embraces the importance of these non-helical structures in intermonomeric covalent crosslinking as described earlier (Kagan & Trackman 1991, Kielty et al. 1993, Kivirikko 1995, and Kuznetsova & Leikin 1999). Nucleation proceeds through polymerization to formation of primary substructure of collagen fibrils. In this collagenous pentamer, five monomers are aligned longitudinally to overlap 3.4 D (1 D = 67 nm = 234 aa) with adjacent monomer (1 D stagger). If this pentamer is not cyclical, it leads to sheet-like orientation of monomers within mature fibrils. In fibrils having the cyclic orientation of collagen monomers, both with and without microfibrillar substructure, the first and the fifth monomer overlaps 0.4 D (4 D stagger) and

thereby closes the helical cylinder. This mechanism of cooperative helical polymerization of collagen monomers is congruent with *in vitro* assembly of tubulin to microtubules and G-actin to actin filaments, which led to the initial proposal of nucleation-growth mechanism for collagen fibril assembly. In cooperative reactions weak self-association must precede a stronger one enabling the system to restrict the number of growing sites and generate a small number of large biomolecular assemblies rather than a large number of small aggregates (Wood 1960a, Oosawa & Kasai 1962, and Gaskin et al. 1974). The sheet-like or cyclic pentamers are elongated furthermore by addition of monomers to present a 0.6 D (40 nm) gap between coaxial and D-stagger interaction to neighboring molecules (Smith 1968, Na et al. 1986a, and Miller & Gay 1987). Elongation, as well as nucleation, is driven by negative free energy change when the solvent molecules are released from the overlapping surface of collagen monomers (Na et al. 1986a, and Na et al. 1986b).

The models of longitudinal packing, of monomers and the pentamer based primary structure, have been mostly agreed for several decades. However, further diameter growth of these intermediates to collagen fibrils and the lateral packing of monomers within them have been under intensive discussion and some different point of views have been expressed. These partly conflicting models have mainly been based on even re-evaluated X-ray diffraction data and electron microscopical evidence concerning collagen fibrils from different origin tissues, and in some cases with further computer assisted theoretical modeling. Both assembly kinetics and the detailed lateral orientation of monomers have been discussed about fibrils having the cyclic monomer orientation without microfibrillar substructure. For the fibrils having microfibrillar substructure or sheet like orientation of collagen monomers, predominantly different topological proposals without kinetic speculations about the mechanism of fibril propagation have been presented. At the moment consensus view includes quasi-hexagonal packing of monomers in triclinic cell units. The arrangement of molecules within the unit cell is, as mentioned above, either sheet-like or cyclic, generating crystalline or less organized and more flexible liquid crystalline nature having fibril domains (Prockop & Fertala 1998, and Wess et al. 1998). However, when this generally established crystal structure based on x-ray-diffraction data (Hulmes & Miller 1979, Fraser et al. 1987, and Wess et al. 1995) was compared to observation about collagen type I fibrils *in vivo* some conflicting features were discovered (Prockop & Fertala 1998). The most important features are the roundness of fibrils, even fibrils having diameter as small as 40 nm, and the fibril growth from symmetrical and paraboloidal tips (Kadler et al. 1990b). The former indicates the presence of cyclic orientation of monomers around pentameric microfibrillar core without actual microfibrillar substructure. On the contrary, questions are raised on the stepwise increase (≈ 8 nm) in fibril diameter observed during the growth and development (Craig & Parry 1981), and the microscopical evidence for existence of microfibrils as substructure in many but not in all fibrils (Piez & Trus 1981).

Based on these inconsistent observations, two main theoretical strategies to illustrate the formation of supramolecular aggregates having the cyclic orientation of monomers have been presented. The first branch explains the growth of fibrils by the step by step addition of monomers to surround the pentameric, structural core that is contemporaneously elongated to rod-like fibril. Starting from this premise, two computer-generated models of fibril growth have been proposed. Silver et al. (1992) have developed a model for tip

growth based on observations made in *de novo* at 37 °C about collagen type I fibril growth from near paraboloidal symmetrical tips. This theory takes into account circular cross-section of fibrils down to the tip of < 25 nm (Kadler et al. 1990a, and Holmes et al. 1992), the decrease in mass in both tips and the constant contour when fibrils grew (Holmes et al. 1992). This model includes distinctive spiral or helical N-terminal nucleus at each pointed end of a growing fibril from which the growth proceeds in both directions and the fibril shape would be determined by different binding site with different accretion rates. According to another model (Parkinson et al. 1994, and 1995) fibrils are formed by diffusion-limited aggregation in which the fibril assembly is limited by the rate of diffusion of monomer to the fibril surface and the number of binding sites available increases with the size of the aggregate. Similar to Silver´s presentation, this theory explain the fibril growth from paraboloidal tips, a preference for tip growth and a linear relationship between mass and distance from the tip (Prockop & Fertala 1998). The other branch of theories concerning the fibrils having the cyclic orientation of monomers, first presented by Smith (1968), explain the lateral growth of aggregates by the compression of primary emerged microfibrils, which leads to the formation of thick fibrillar bundles, in other words to "mature" collagen fibrils having the microfibrillar substructure. This five-stranded microfibril model has gained wide acceptance being consistent with some X-ray diffraction data and with a quite wide electron microscopical evidence concerning filamentous structure of native fibrils (Piez & Trus 1981). This model explains polymorphic collagen fibril structures reported by Piez & Miller (1974) and Doyle et al. (1974) as well as thin, but still D-periodic filament demonstrated by Veis et al. (1979). Later Trus & Piez (1980, 1981) modified Smith's presentation and proposed a compromise between the compressed microfibril model and the hexagonal lattice structure originally based on sheet-like structure of pentamers that was presented by Hulmes & Miller (1979). This idea was earlier suggested generally by Bailey et al. (1973) and Katz & Li (1973) and it was later re-evaluated and specified due to impressive x-ray data supporting the presentation of Hulmes & Miller in 1979 (Piez & Trus 1981).

In addition to the X-ray diffraction data about collagen crystals and microscopical data about fibrils in tissues, *in vitro* experiments concerning fibril assembly from acid extracted collagen monomers support on the other hand the existence of microfibrils as fibrillar substructure and sheet-like or helical orientation of monomers without microfibrillar substructure within fibrils. During the end of the lag phase and the early growth phase, neutralized and thermally induced collagen monomers form the D-periodic fibrils from 1 to 20 μm in length. The fibril diameter varies between 20 and 200 nm depending on the induction temperature in the experiment (Wood 1960a, Wood & Keech 1960, Holmes & Chapman 1979, and Holmes et al. 1986). Some indication about endogenous fibril diameter control exists also *in vitro*. The limiting fibril diameter seems to occur when about 20 % of collagen monomers have assembled to fibrils (Bard & Chapman 1973). Since then the assembly continues at the ends of existing aggregates. In addition, some early fibrils showed well defined shape with the constant size of 90 D-periods (≈ 6 μm) in length and with maximal cross-section containing about 160 molecules (Holmes & Chapman 1979). In addition to banded fibrils and similar to cold-induced disassembly reaction, some unbanded fibrils of 2-4 nm diameters have also been observed as intermediates during fibril assembly (Gelman et al. 1979a,

Veis et al. 1979, Holmes & Chapman 1979, Na et al. 1986a, and Holmes et al. 1986). In some experiments the non-banded kinetically stable microfibrils having diameter about 10-20 nm occurred during the lag phase and therefore concluded that the D-banded fibrils are formed by lateral and parallel fusion of these filaments during the sigmoidal increase of turbidity leading to microfibrillar substructure within fibrils. The formation of these nonbanded intermediates during lag phase is considered to be undetectable because of the low concentration and the small diameters of these structures (Williams et al. 1978, Na et al. 1986a, and Na et al. 1986b). On the other hand, the formation of unbanded fibrils were later proposed to be due to the order of warming and neutralizing that affects the conformation of terminal telopeptides, and therefore, produces "incorrectly" packed fibrils rather than indicative of the existence of nonbanded microfibrils as intermediates. According to this the D-periodic fibrils are not formed if the collagen solution is neutralized before thermal induction of fibril assembly (Holmes et al. 1986, and Kadler et al. 1996). However, this proposal is not congruent with observation made about heterotypic fibril assembly. Blaschke and co-workers (2000) reported the banded pattern of fibrils containing collagen types II, IX, and XI, despite the cool and neutral step in the beginning of experiment. Altogether the existence of different kind of intermediates between the monomers, the microfibrils, and the banded fibrils vary between the observations of different research groups. The intermediate´s structural relationship to mature D-banded fibrils and monomer orientation within them is not clear (Silver et al. 1979, Silver & Trelstad 1980, Silver 1981, Gelman & Piez 1980, Bernengo et al. 1983, and Na et al. 1986a). It is also necessary to mention that, despite the fact that, the collagen monomers contain practically all the information needed for fibrillogenesis the process in probably controlled by several enzymatic and cellular mechanisms. The relation of collagen fibrils to collagen binding integrins is not known.

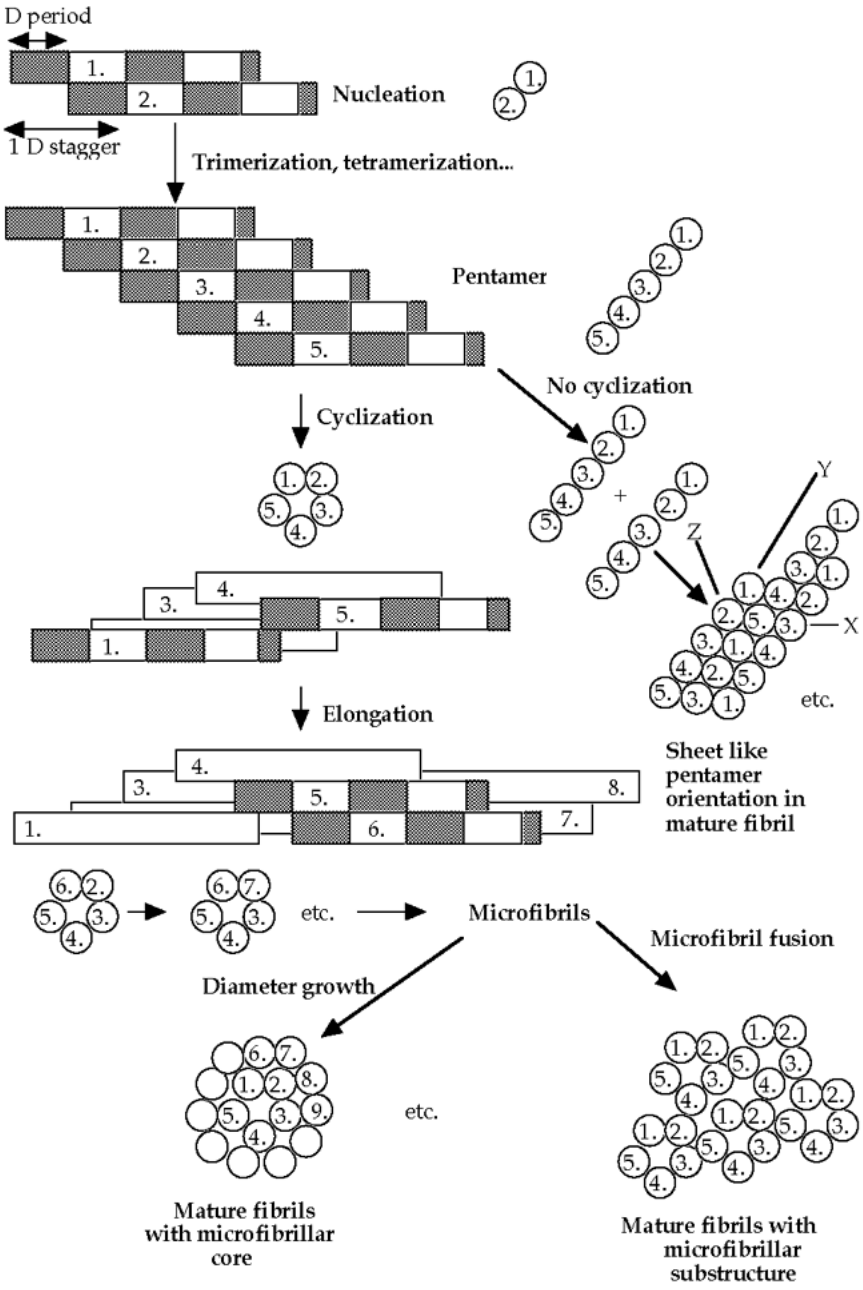


FIGURE 1 Summary of main theories about collagenous fibrillogenesis and monomer orientation within mature collagen fibrils based on X-ray crystallographic and electron microscopical data. Fibril formation starts with nucleation and leads through oligomerization to the formation of a pentamer. If the pentamer is closed as a cylinder, it is further elongated to microfibril. These microfibrillar structures can grow in diameter either by attachment of monomers around the microfibrillar core or by microfibrillar fusion together to mature fibrils. If a pentamer is not cyclized, it leads sheet like monomer orientation in fibrils. In this process elongation and diameter growth propagate attachment of pentamers together to existing sheet. For references see text above.

2.1.2 Non-fibrillar collagens

Non-fibril-forming collagens are a structurally and functionally diverse subgroup if compared to fibril-forming collagens. Instead of being structural components of the fibrils, they fulfill a great variety of biological functions. Non-fibrillar collagens have one or more interruptions in the Gly-X-Y repetitive sequence and based on structural and functional characteristics they are divided to six subfamilies: collagens that form network-like structures (types IV, VIII, and X), fibril-associated collagens with interrupted triple helices (FACIT; types IX, XII, XIV, XVI, XIX, XX), beaded filaments forming collagen (type VI), anchoring fibrils forming collagen (type VII), transmembrane collagens also known as membrane-associated collagens with interrupted triple-helices (MACIT; types XIII, XVII, XVV, XXIII) and a group called MULTIPLEXINs (types XV and XVIII) i.e. collagens with multiple interruptions in the triple helical domain (Myllyharju & Kivirikko, 2001, Koch et al. 2001, and Banyard et al. 2003). In addition, organization and characterization of the human collagen type XXI gene (*COL21A1*), which seems resemble members in FACIT collagen gene family has recently been presented (Chou & Li 2002). More detailed description about collagen type IV, and particularly about collagen types XIII and XVII is seen in the following.

2.1.2.1 Network-forming collagens - collagen IV as prototype

Discovery of collagen type IV was the first step in classifying non-fibrillar collagenous compounds and thereby creating bases to the molecular section originally called "group II" collagens (Miller & Gay 1987). This prototype molecule within non-fibrillar collagen subfamily is widely distributed as the major component of all basement membranes. In these sheet-like structures type IV collagen molecules associate with laminin, entactin/nidogen and heparin sulfate proteoglycans to generate an elaborate network that compartmentalize and create functional molecular meshworks. The most common type IV molecule is a heterotrimer composed of two α1(IV) chains (gene *COL4A1*; P02462) and one α2(IV) chain with a combination existing abundantly in mesangial, vascular and tubular matrix membranes (gene *COL4A2*; P08572; Timpl 1989, Yurchenco & O´Rear 1994, and Timpl 1996), In addition, in functionally specialized basement membranes more tissue specific α3(IV), (gene *COL4A3*; X80031), α4(IV) (gene *COL4A4*; X81053), α5(IV) (gene *COL4A5*; AH006316), and α6(IV) (gene *COL4A6*; AH003699) chains occur in the trimer composition (Hudson et al. 1993, and Ninomiya et al. 1995). Especially high amounts of α3(IV), α4(IV), α5(IV) chain expression have been reported in kidney glomerulus and lungs. Generally, α3(IV) and α4(IV) chains always seem to be co-expressed together and appear only in the α5(IV) positive basement membranes (Miner & Sanes 1994). On the other hand, chain α6(IV) is absent in glomerular type IV monomers, but is expressed in matrix structures covering epidermal membranes, Bowman´s capsule, renal tubules, smooth muscle cells and adipocytes (Ninomiya et al. 1995). The total number of different type IV isoforms is not known. Based on structural characters the relatively homologous α(IV) chains can be divided into α1 chain-like (α1(IV), α3(IV), α5(IV)) and α2 chain-like class (α2(IV), α4(IV), α6(IV)). Each extensively hydroxylated and glycosylated α(IV) chain in monomer form is segmented to a short N-terminal cysteine-rich (≈ 15 aa; 7S) domain, a central triple-helical

domain (≈ 1400 aa) interrupted by short non-collagenous segments, and a C-terminal non-collagenous domain (≈ 230 aa; NC1; Hudson et al. 1993, and Prockop & Kivirikko 1995). Instead of fibrils, type IV monomers are self-assembled into network-like structures based on several intermolecular interfaces. Monomers can associate at the C-termini (NC1-NC1 interaction) to form a dimer stabilized by interchain disulfide bonds and at the N-termini to form a tetramer (7S-7S-7S-7S interaction) stabilized both by intra- and intermolecular disulfide bonds. In addition to terminal interactions, flexible noncollagenous interruptions in collagenous domains allow triple helical domain to interact with highly conserved NC1 domain and form supercoiled structures with adjacent monomers (Hudson et al. 1993). The human α1(IV) chains as a main component of type IV monomers contain one GFPGER-motif which might act after proline hydroxylation as a binding site for collagen receptor integrins (P02462; Knight et al. 1998, Knight et al. 2000, Xu et al. 2000, and Zhang et al. 2003). In addition, this amino acid sequence can be found in some minor human α chain types, i.e. in α3(IV), α4(IV), and α5(IV) peptides. Interestingly, disturbed collagen type IV network based matrix interactions can be caused by dozens of mutations in the minor α(IV) chain types coding genes (*COL4A3*-*COL4A6*), but not in the most abundantly expressed α1(IV) and α2(IV) chains. More than 50 mutations has been reported in *COL4A5* leading to clinical condition called Alport syndrome, i.e. a progressive kidney disease caused by structural changes in glomerural basement membranes. In addition, a lethal autoimmune disease targeted to the NC1 domains of type IV molecules in glomerural and alveolar basement membranes called Goodpasture syndrome is known (Hudson et al. 1993, and Prockop & Kivirikko 1995).

2.1.2.2 Transmembrane collagens

The subfamily of transmembrane collagens, also known as membrane-associated collagens with interrupted triple-helices (MACITs), covers collagen type XIII, hemidesmosomal collagen type XVII and newcomers namely collagen types XVV and XXIII. These nonfibrillar collagens are not structurally homologous, except that they have an anchoring transmembrane domain. For neuronal collagen type XVV (CLAC-P; collagen-like Alzheimer amyloid plaque component) see Hashimoto et al. 2002 and for XXIII in metastic tumor cells see Banyard et al. 2003. In some presentations ectodysplacin A (Elomaa et al. 2001, macrophage scavenger I and II type receptors (Krieger & Herz 1994) and MARCO (Sankala et al. 2002) proteins with collagenous domains are included in this family despite the fact their role as extracellular structural molecule is under discussion. The following review concerns collagen types XIII and XVII with some analogous characters in their biology. Their structure is presented schematically in Fig. 2.

Collagen type XIII is widely expressed. It has been localized to many sites of the cell-matrix interactions and at some cell-cell interaction sites (Peltonen et al. 1999, Sandberg-Lall et al. 2000, Hägg et al. 2001, and Sund et al. 2001). The high degree of colocalization with E-cadherin suggests that type XIII collagen is very likely to be closely associated with adherens type junctions in normal human skin and cultured keratinocytes (Peltonen et al. 1999). Collagen type XIII was found to be widely expressed in ocular tissues both in fetal and adult human. The strongest signals occurred in the optic nerve bundles and in the ganglion cell layer of the retina. Other notable locations containing collagen

type XIII included the developing ciliary smooth muscle, the posterior two-thirds of the corneal stroma and the striated extraocular muscles. Low level signals were also detected in the blood vessel walls and mesenchymal cells of the other ocular tissues (Sandberg-Lall et al. 2000). Further, collagen type XIII was found in a range of integrin-mediated adherens junctions including the myotendinous junctions and costameres of skeletal muscle as well as in many cell-basement membrane interfaces. Some cell-cell adhesions were found to contain collagen type XIII, most notably the intercalated discs in the heart (Hägg et al. 2001). Sun et al. (2001) studied collagen type XIII expression and spatio-temporal localization during mouse fetal development. Collagen type XIII mRNAs were expressed at a constant rate during development, with an increase of expression towards birth. Strong collagen type XIII expression was detected in the central and peripheral nervous systems of the developing mouse fetus in mid-gestation. Cultured primary neurons also expressed this collagen, and it was found to enhance neurite outgrowth. Also in this study, strong expression during early development was also detected in the heart, with localization to cell-cell contacts and accentuation in the perinatally intercalated discs. During late fetal development, collagen type XIII was observed in many tissues, including cartilage, bone, skeletal muscle, lung, intestine and skin. Notable structures lacking collagen type XIII were the endothelia of most blood vessels and the endocardium. Its initially unique staining pattern began to concentrate in the same adhesive structures where it exists in adult tissues, and started to resemble that of the β1 integrin subunit and vinculin during late intrauterine development and in the perinatal period.

The size of gene coding α1(XIII) chain, *COL13A1*, varies between 135-138 kb in size. It consists of 42 exons located in chromosome 10 (Shows et al. 1989, Tikka et al. 1991, and Kvist et al. 1999). The collagen type XIII monomer, about 150 nm in length, is situated in the cell plasma membrane with a type II orientation having homotrimeric α1(XIII) chain composition with three stable triple helical collagenous domains. The structure seems to be highly conserved. The overall identity between human and predicted mouse polypeptides is 90 % and their similarity 94 %. For human α1(XIII) and mouse α1(XIII) chain coding sequences see access numbers NP_543005 and U30292, respectively. In human, this rod-like monomer has a short N-terminal cytosolic domain (38 aa), a single hydrophobic transmembrane domain (23 aa), and a large (≈ 650 aa), C-terminal, mainly collagenous ectodomain (Pihlajaniemi et al. 1987, Pihlajaniemi & Tamminen, 1990, Hägg et al. 1998 and Snellman et al. 2000). Typically, type II transmembrane proteins that have intracellular N-terminal portions and extracellular C-terminal portions including collagen type XIII polypeptide, lack the N-terminal signal peptide (Hägg et al. 1998 and Singer 1990). The most amino terminally situated non-collagenous domains, NC1, cover the intracellular domain, transmembrane domain and first 60 residues from the extracellular part adjacent to the plasma membrane. The rest of extracellular part contains three collagenous sequences, COL1-COL3, with sizes of 57-104, 172, and 184-235 residues, respectively. These triple helical areas are interrupted further with noncollagenous sequences, NC2-NC4, with sizes of 12-34, 22, and 13-18 residues, respectively (Hägg et al. 1998). In human, collagen type XIII the primary transcripts from10 exons ungergo intensive and complex alternative splicing, whichresults in prominent variation in the length of Col1, NC2, COL3, and NC4 domains (Pihlajaniemi & Tamminen 1990, Tikka et al. 1991, Juvonen et al. 1992, Juvonen & Pihlajaniemi, 1992, Juvonen et al. 1993, Peltonen et al. 1997,

and Kvist et al.1999). As a result of this, at least 19 human α1(XIII) chain isoforms are known (NCBI). According to Tu et al. (2002) about 72 % of prolines and 30 % lysines in the ectodomain are hydroxylated resembling those reported for other collagens. The α1(XIII) chain contains several cysteines, which can form either intrachain or interchain disulfide bonds. There are four cysteins in NC1 domain, namely two in the transmembrane portion and two at the junction of NC1 and COL1 (Hägg et al. 1998). Some of these NC1 domain residues participate in the formation of interchain disulfide bonds. Additional cysteine residues in the COL1 and NC2 domains are as well suggested to participate in interchain bonding. In contrast cysteine residues occurring in the NC4 domain form intrachain bonds (Snellman et al. 2000).

Studies with surface plasmon resonance technique and ELISA solid phase assay revealed that human recombinant collagen type XIII ectodomain interacts with immobilized heparin ($K_d \approx 1.8$ nM) fibronectin ($K_d \approx 2.5$ nM), nidogen-2 ($K_d \approx 4.5$ nM), and perlecan ($K_d \approx 9$ nM). In these studies binding to other basement membrane components like vitronectin, a laminin-1-nidogen-1 complex, nidogen-1, fibulin-1, fibulin-2, and collagen types I, III, IV and VI was not observed (Tu et al. 2002). The α1(XIII) chain sequences reported this far, does not contain any known integrin recognition motifs and the biological importance of collagen type XIII is not known. To shed light on this, a mouse strain that expresses an N-terminally altered form of type XIII was established. This was done through site-specific deletion of exon 1 sequences from embryonic stem cells. No phenotypic abnormalities were detected on inspection of mutant embryos or new born mutant mice. Furthermore, the homozygous mice for allelic change showed no changes in their growth, behavior, or reproduction compared to normal littermates. However in mutant mice, the fibroblasts derived from embryos show 8-15 % adherence to culture plates than controls. On collagen type IV surface the difference was even more dramatic. In addition, according to histological examination by light microscopy, some changes in the skeletal muscle were observed. Some muscle fibers appeared uneven and smaller size with a wawy sarcolemma. In addition, adjacent fibers in appeared to be more loosely attached to each other if compared to wild-type animals. According to more detailed ultrastructural analysis by electron microscopy revealed vacuolization, accumulation and enlargement mitochondria, and disorganization of myofilaments and Z-bands in mutant animals. Defects stated were detected more frequently and were more pronounced in older mice, suggesting a progressive condition. Additionally, acute physical exercise was found to induce muscle cell damage more frequently in quadriceps femoris and gastrocnemius muscles of mutants than wild mice. In comparison to wild mice, mutant runners had more numerous fibers undergoing degeneration and more intense inflammation. Alltogether, the results so far suggest that collagen type XIII participates at least in linkage between the muscle fiber and the basement membrane (Kvist et al. 2001). No pathological condition related to collagen type XIII or it's relation to collagen binding integrins is known.

Collagen type XVII (HD4, 180-kDa bullous pemphigoid antigen, BP180, BPAG2) homotrimer is typically a major structural component of plasma membrane associated hemidesmosomes in the basal surface of stratified epithelial cells like basal keratinocytes. These structures projects into the basal lamina and links keratin intermediate filaments of stratified epithelia to components of the extracellular matrix (Nishizawa et al. 1993, and Giudice et al.

1992). According to immunoelectron microscopy, it seems that extracellular region of collagen type XVII monomer spans the lamina lucida inserting into the lamina dense, though the C-terminal part colocalizing with laminin-5 (Bedane et al. 1997). However, also some structures at the cell-tissue interfaces other than hemidesmosomes may also contain collagen XVII (Jones et al. 1998 and Borradori & Sonnerberg 1999). Significant level of expression of collagen type XVII , besides in epithelial keratinocytes, also in a variety of tissues with predominant epithelial component, such as mammary, salivary, and thyroid glands, colon, prostate, testis, placenta, thymus and colon (Aho & Uitto 1999). The cDNA of α1(XVII) chain predicts collagen type XVII monomer to be similar to collagen type XIII, the type II transmembrane protein consisting of 1497 amino acids. For collagen type XVII sequence see access number CAC00589. Sequence seems to include putative NH_2-terminal globular intracellular domain of 466 amino acids with disulphide bonds, transmembrane domain of 23 residues and a COOH-terminal extracellular domain of 1008 amino acids. This C-terminal half contains area of 916 residues with 15 collagenous domains with Gly-X-Y sequence repeats encompassing a majority of region and is capable of forming collagen trimer analogous to intact endogenous molecule. These N-glycosylated triple helical areas are variable in length the size varying from 15 to 242 amino acids (Giudice et al. 1992, Balding et al. 1997, and Schäcke et al. 1998). Triple helical area of ectodomain remains stable alignment and is resistant to proteolysis at temperatures above 40 °C (Schäcke et al. 1998). The rod-like sections of trimer correspond to the coiled-coil and collagen-like triple helices (Balding et al. 1997). Collagenous motifs are separated from one to another by short stretches of non-collagen sequences (Giudice et al. 1992) which further allows flexibility of the protein for efficient ligand interaction (Balding et al. 1997, and Schäcke et al. 1998).

Collagen type XVII seems to interact in hemidesmosomes with integrins. Hopkinson et al. (1995) suggested that the NH_2-terminal domain of collagen type XVII determines polarization of a molecule while the noncollagenous motifs in the extracellular domain stabilize its interactions with other hemidesmosomal components, such as some α6 subunit containing integrin. The results by Borradori and co-workers (1997) showed that the cytoplasmic domain of collagen type XVII contains sufficient information for the recruitment of the protein into hemidesmosomes because removal of the extracellular and transmembrane domains does not abolish targeting. Further, the studies with chimeric proteins revealed that localization of collagen type XVII in hemidesmosomes is mediated at regions located within the central part and at the NH_2-terminus of the cytoplasmic domain containing 265 residues. The colocalization with α6β4 integrin was established. In this process especially the importance of β4 subunit's cytoplasmic domain was observed. Hopkinson et al. (1998) showed interaction with integrin was not only mediated by collagen type XVII, but also necessary in the first place for hemidesmosome formation.

Moreover from the former hints arose about collagen type XVII functioning as cell-matrix adhesion molecule, which are collagen type XVII related diseases characterized by blistering of skin due to fragility at the dermal-epidermal junction. Data indicates that a humoral autoimmune response directed against the ectodomain of collagen type XVII is responsible for the detachment of basal keratinocytes from the basal lamina in acquired human diseases called bullous pemphigoid and herpes gestatinonis (Giudice et

al. 1993, and Liu et al. 1993, and Giudice et al. 1995). Generalized atrophic benign epidermolysis bullosa (GABEB) is a form of nonlethal junctional epidermolysis bullosa characterized by universal alopecia and atrophy of the skin belonging in the group of genodermatoses. The studies reveal that the collagen type XVII is deficient and collagen type XVII coding mRNA is reduced in this disease (Jonkman et al. 1995). Patients with GABEB seems have premature termination codons on both alleles in *COL17A1* gene (McGrath et al. 1995). Collagen type XVII relation to collagen binding integrins is not known.

Interestingly, in addition to anchored transmembrane form of collagen types XIII and XVII these polypeptides occur also as shorter soluble forms. In the first place the soluble ectodomain of these collagens was found from culture medium of wild-type keratinocytes and immortalized HaCat cells (Schäcke et al. 1998, and Peltonen et al. 1999). The biological importance of this phenomenon is unknown. Numerous observations concerning particularly collagen type XVII support the proposition that these soluble forms of collagen molecules are released in post-translational proteolysis by cell surface oriented secretases or sheddases from intact transmembrane monomer. First, the apparent size of soluble collagen type XVII ectodomain (120 kDa) corresponds to the size of digestion product of full-length collagen type XVII molecule (Schäcke et al. 1998). In some cases, an even shorter fragment around 90-100 kDa has been observed (Hirako et al. 1998). Secondly, both antibodies against 205 with most carboxyl-terminal residues and antibodies against NC16 domain adjacent to plasma membrane recognize the soluble ectodomain other than antibodies against the intracellular part. In addition, the proteolytical sensitivity of the soluble form for enzymes like collagenase, pepsin, and N-glycosidase F are comparable to the sensitivity of intact ectodomain. Importantly, the soluble ectodomain is not found from *COL17A1* nullizygote keratinocyte cell cultures and according to cDNA probing studies both polypeptides seems to be translated from the same mRNA template (Schäcke et al. 1998).

In collagen type XIII, the cleavage site of the secreted ectodomain is before the RRRR motif in position 105-108 in NC2 area, which indicates that one or more furin-like proteinases are involved in this process (Nakayama 1997, Snellman et al. 2000b). Seven distinct proprotein convertases of this furin family have been identified in mammalian species expressed in broad range tissues and cell lines. These enzymes has been shown to be responsible for conversion of precursors in a wide variety of proteins, including growth factors, serum proteins, proteases of the blood-clotting and complement systems, matrix metalloproteinases, receptors like integrins, viral-envelope glycoproteins and bacterial exotoxins into their biologically active forms (Nakayama, 1997). Importantly, the type XVII shedding was enhanced by phorbol esters and the synthetic furin inhibitor, decanoyl-RVKR-chloromethyl ketone, does inhibit the formation of released forms of both collagen types XIII and XVII (Schäcke et al. 1998, Snellman et al. 2000b, and Franzke et al. 2002). Two kind of putative furin activation site containing enzyme based mechanisms in this process are proposed. Furin either activates the genuine collagen converting proteinase or furin processes collagen ectodomain directly. The involvement of matrix metalloproteinases expressed in keratinocytes like MMP-9 and MMP-14 has been speculated to take part collagen type XVII processing (Franzke et al. 2002). The involvement of TACE, ADAM-9, and ADAM-10 was published recently (Schäcke et al. 1998). The importance of calcium in collagen type XVII shedding was shown indicating that also calcium dependent proteinases are involved in

the processing (Schäcke et al. 1998). Interestingly, it was shown that also the heparin can inhibit type XIII ectodomain shedding (Tu et al. 2002).

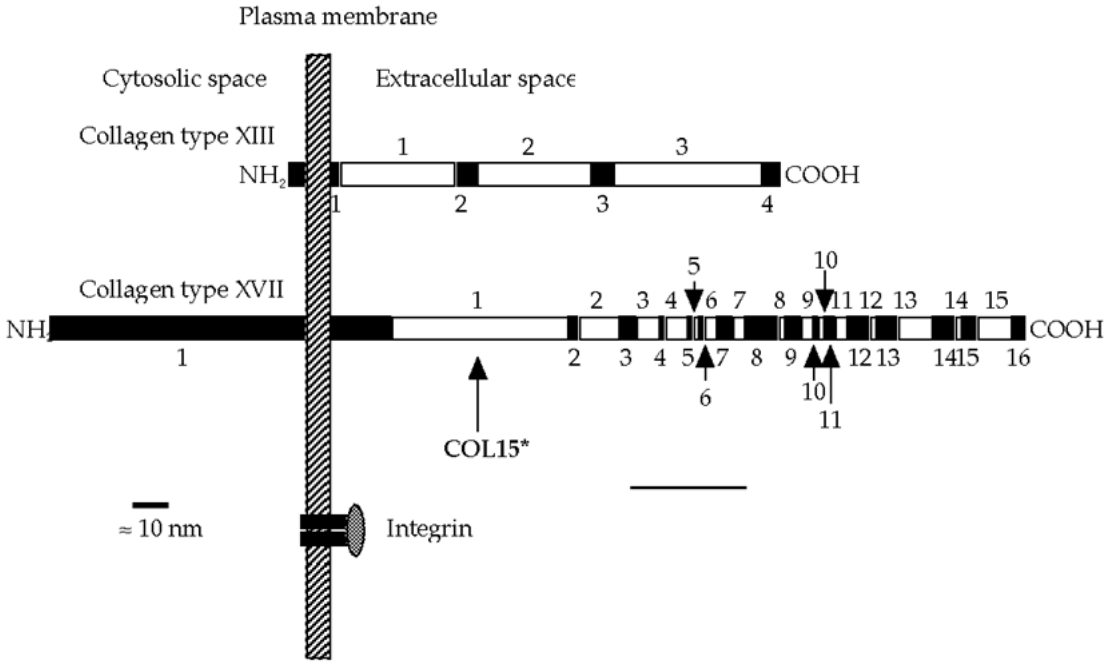


FIGURE 2 Schematic comparison of transmembrane collagen types XIII and XVII. The numbering of the non-collagenous (black stretch) and collagenous (white stretch) domains are indicated by numerals below and above, respectively. Exceptionally, in some publications, as well as in this thesis, the numbering of XVII domains has started from COOH-terminus. According this, the COL15 domain of collagen type XVII studied, is indicated by asterix (*). This presentation is modified from publications by Pihlajaniemi & Tamminen, 1990, Tasanen et al., 2000, and Tu et al., 2002.

2.2 Family of integrins

The integrin-type receptors from invertebrates, including at least phylas like sponges, cnidarians, nematodes, arthropods, and echinoderms, to vertebrates have had an important role in the development of these life forms as multicellular organisms and it is thought that all metazoan cells have at least one integrin receptor. In addition, the integrin subunits found from different levels of phylogetic tree show remarkably structural and functional similarity (Müller 1998 and Burke 1999). However, the receptor diversity varies widely between animal generas. For example, instead of great integrin subunit variety in mammals, the *Drosophila* and *Caenorhabditis* genomes encode only five and two integrin α and β subunits, respectively (Humphries 2000). Integrins are heterodimeric, transmembrane proteins which interact with a great variety of extracellular matrix components, complement proteins, bacterial and viral proteins, coagulation and fibrinolytic factors and other cells while their intracellular parts interact with the cytoskeleton. By these means integrins allow dynamic cell-cell and cell-matrix adhesion as well as the processes of signal transduction included in embryological development, hemostasis, thrombosis, wound healing, immune and non-immune defence mechanisms (Hynes 1987 and Haas 1994). In addition to normal physiological processes while maintaining tissue integrity, they are involved in initiation and/or progression of pathological conditions like chronic inflammation, invasion of cancer and tumor metastasis to name but a few (Heino 1993 and 1996). This functionally versatile receptor family can be divided into subgroups based on shared α or β chains (like β1 integrins), shared ligand recognition properties (like RGD dependent or RGD independent integrins), or shared structural features of the α subunits (like αI domain containing and non αI domain containing integrins) as in the following.

2.2.1 Integrins in the beginning

The first steps in the history of knowledge concerning integrins are placed in early 1980's. Two main approaches have been used to isolate and detect these receptors, namely immunoselection with antibodies that block cell adhesion and affinity chromatography with extracellular ligands (Hynes, 1987). An important stage in the discovery of integrin family was idenfication of fibronectin as cell adhesion supporting substrate. Further, the basis of this interaction was allocated to tetrapeptide containing arginyl-glycyl-aspartyl-serine residues, later known generally as RGD motif (Pierschbacher & Ruoslahti 1984). Almost concurrently, the thrombin-induced aggregation of platelets was shown to be mediated by two non-covalently to transmembrane complex linked glycoproteins existing in ratio of 1:1. Data showed that these monomeric proteins, called in the first place IIb and IIIa, form a heterodimeric receptor and the dimer formation depends upon the presence of Ca^{2+} (Jennings & Phillips 1982). In addition to fibronectin, the molecular interaction between RGD motif containing fibrinogen, von Willebrandt factor, vitronectin and this IIbIIIa receptor, later termed CD41/CD61 or αIIbβ3 integrin, was established (Ruggeri et al. 1982, Bennett et al. 1983, Gardner & Hynes 1985, and Pytela et al. 1986). In the case of collagen receptor integrins, the studies dealing with lymphoid and myeloid cells exposed the existence of two sets of homologous dimers on the

surface of activated T lymphocytes very late after activation by antigen or mitogen, termed as very late antigen-1 (VLA-1) and very late antigen-2 (VLA-2; Hemler et al. 1985, and Hemler & Jacobson 1987). They are later known as α1β1 and α2β1 integrins, respectively. The finding that in leukocytes a set of different larger subunits, shared the common smaller subunit, was the beginning of cell-cell adhesion mediating integrin discovery. These dimers identified by utilizating certain monoclonal antibodies were called LFA-1 (later named to CD11a/CD18 or αLβ2 integrin), Mac-1 (CD11b/CD18, CR3, αMβ2), and p150,95 (CD11c/CD18, CR4, αXβ2; Springer et al. 1985, and Anderson & Springer 1987).

After these proteins were cloned, it was obvious that these dimers are members of same receptor family (Albelda & Buck 1990, Hemler 1990 and Ruoslahti 1991). Before this, Tamkun et al. (1986) had proposed the name "integrin" for protein dimer complex they had characterized from chicken cells to denote its role as an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton. Later this name was given for the intery "integrating" protein group. The integrin nomenclature used nowadays was presented in a review by Hynes (1987). In this review, the smaller disulphide-rich subunits were refered as β subunits and the dimer partners as α subunits. Later on, it was discovered that different subunit combinations have different ligand binding spesificity and this feature is determined by both subunits (for example see Vogel et al. 1990 and Bodary et al. 1990). In addition, the different subunit splice variants were discovered. Van Kuppefelt et al. (1989) found the difference in β3 subunit and suggested that the difference between the cytoplasmic domains of these subunits arose as a result of an alternative mRNA splicing and these cytoplasmic domains may provide alternative means for the integrin subunit to interact with cytoskeletal components.

The first evidence about inside-out signaling was presented via conformational changes in αIIbβ3 integrin. These changes include alterations in the state of activation of this receptor that can affect the binding of adhesive proteins, and the formation of platelet aggregates (Phillips et al. 1991). At the same time first hints about outside-in signaling was found. Kornberg et al. (1991) and Guan et al. (1991) postulated that the integrin-stimulated tyrosine phosphorylation of pp120 and pp130 proteins may reflect part of an important signal transduction process between the extracellular matrix and the cell interior and this may be involved in the responses of cell attachment. These findings as keystones gave the beginning to live research branches concerning integrins. As example of vividness, the integrins studying groups have produced over 3000 scientific publications during the past year. About every 10th of these dealt collagen binding integrins (PubMed).

2.2.2 Integrins presently

Currently, 24 different integrin dimer combinations have been found in mammals formed by 18 α subunits (α1-α11, αE, αL, αM, αX, αD, αV, αIIb; 120-180 kDa) and 8 β subunits (β1-β8; 90-110 kDa). Eighteen of the integrin receptors are cell-matrix interaction mediating structures while the rest participate in cell-cell adhesion primary with counter receptors (ICAMs, VCAM-1, and MadCAM-1). The cell-matrix interaction integrins are common to all nucleated cells and they play important roles in platelet function as well. The

cell-cell interactions mediating integrins are largely confined to cells in the immune system. A minority of integrins, like αMβ2,can mediate both interactions. The integrins display both specificity and redundancy with overlapping functions. As example of specificity it can be mentioned α5β1 dimer which has only one characterized natural ligand, namely the central cell-binding domain of fibronectin. Instead, αVβ3dimer has several apparently dissimilar ligands, including fibrinogen, vitronectin, fibronectin, von Willebrand factor, osteopontin, and bone sialoprotein. Furthermore, the same region in one adhesion protein can be recognized by several different integrins. As an example, the cell-binding domain of fibronectin is recognized by α5β1, α3β1, αVβ1, αVβ3, αIIbβ3, and αVβ6 integrins (Garratt & Humpries 1995 and Humphries 2000). The known ligands of αI domain containing integrins are summarized in Table 1. In addition to known dimer combinations, more functional variation is achieved by expression of more or less tissue specific alternatively spliced mRNAs coding the integrin subunits. Both α and β subunit isoforms may contain differently spliced extracellular and cytoplasmic domains as a mechanism to regulate ligand binding and signaling events (Melker & Sonnenberg 1999).

2.2.3 The collagen binding integrins

Nowadays, four native collagen binding integrins are known, namely α1β1 (VLA-1; CD49a/CD29), α2β1 (VLA-2; CD49b/CD29; GPIa/IIa; ECMRII), α10β1, and α11β1 dimers (Kramer & Marks 1989, DiPersio et al. 1995, Camper et al. 1998, and Velling et al. 1999). In addition, a widely distributed α3β1 integrin (VLA-3; VCA-2; CD49c/CD29; ECMRI; Gap β3) has been reported to interact with a broad spectrum of extracellular ligands including epiligrin, laminin-1, -5, fibronectin and also collagens. However, it does not mediate initial cell adhesion to collagen despite its localization to focal contacts and therefore, it has been suggested that α3β1 dimer may be a secondary receptor with post-cell-adhesion functions (Carter et al. 1991, and DiPersio et al. 1995). In adults α1β1 integrin is expressed mainly in mesenchymal and endothelial tissues. It is a collagen receptor on fibroblasts and microvascular endothelium and found especially abundantly on smooth muscle and liver cells. The α1β1 integrin has dynamic pattern of expression in embryos (Belkin et al. 1990, Duband et al. 1992, and Voight et al. 1995). In addition to structures stated above, it is found at least in small amounts in some developmental stages from neuronal cells, osteogenic cells, platelets, fibroblasts, and keratinocytes (Ignatius & Reichardt, 1988, Belkin et al. 1990, Salter et al. 1995, and Voigt et al. 1995). It is absent in epithelium, and non-activated lymphocytes of the peripheral blood (Voigt et al. 1995). *Vice versa*, the α2β1 dimer has predominantly epithelial distribution, but also found on some cells with non-epithelial origin. It exists on the plasma membranes of fibroblasts, osteogenic cells, chondrocytes, and endothelial cells. It is sole collagen receptor in epithelial cells, and platelets (Giltay et al. 1989, Klein et al. 1991, and Wu & Santoro 1994). The biological phenomena related to collagen binding integrins are reviewed below emphacizeing ligand specificity .

2.2.3.1 Integrin α1β1 - a binding preference to collagen type IV

Riikonen et al. (1995b) reported based on HeLa cell-adhesion studies with function blocking monoclonal antibody SR-84 that α1β1 integrin is a Mg^{2+} and

pH dependent receptor for collagen types I, IV, and V. Ruggiero and co-workers (1996) confirmed, that binding to native, triple-helical collagen type V is mediated by α1β1 integrin in both Mg^{2+} and Mn^{2+} dependent way. It was shown that both α1(V) and α2(V) homotrimers induced human fibrosarcoma cell (HT1080) and human mammary epithelial cell (HBL100) line adhesion but refolded α2(V) chains were more efficient and promoted cell adhesion as well as native collagen V. The α1β1 integrin dependent cell adhesion was more restricted to the heterotrimeric native form of the molecule. Typically, according to solid-phase and inhibition assays with isolated collagen receptors and recombinant αI domains, α1β1 dimer, in contrast to α2β1, favors network like structures forming collagen type IV over fibrillar collagen type I (Kern et al. 1993, Kern & Marcantonio 1998, and Tulla et al. 2001). In the presence of Ca^{2+} and Mg^{2+} the affinity of collagen IV for α1β1 was four-times higher than for α2β1 (Kern et al. 1993, and Kern & Marcantonio 1998). Cell adhesion assays carried out by Loeser et al. (2000) revealed that α1β1 could serve measurably as chondrocyte adhesion receptors also for collagen types II and VI. In cell lines used, the α1β1 was the preferential receptor for collagen type VI while α1β1 attachment to the collagen type II was less obvious. The chondrocyte immortalization with SV40-TAg simian virus results an altered integrin dimer expression if compared to primary cells. Based on these findings it was suggested, that the changes in the relative expression level of α1 subunits may significantly alter the manner in which chondrocytes interact with collagen types II and VI in the extracellular matrix and though expression indicating the differential state and proliferative capacity of the chondrocytes. For α1β1 ligands summarized see Table 1.

Mice carrying a null allele integrin subunit α1 gene are viable, fertile and have no overt phenotype demonstrating that α1β1 integrin is not essentially required for development. However, embryonic fibroblasts or smooth muscle cells derived from knock-out animals are unable to attach to substrata of collagen type IV and laminin along with a deficiency in collagen dependent fibroblast proliferation (Gardner et al. 1996, and Pozzi et al. 1998). *In vitro* analysis of cell spreading and migration assays suggest further that α1β1 dimer is not required for binding to collagen type I (Gardner et al. 1996). Findings are in agreement with the reported binding preferences and indicate possible compensatory role of some other collagen or laminin binding integrins like α3β1 dimer and especially emphasize the important role of α2β1 in collagen type I binding. However, α1β1 null cells do not show any increase in the expression of α2β1 dimer (Hughes 1992, Gardner et al. 1996, and Gardner et al. 1999).

As an indication of collagen type I synthesis feedback inhibitor, α1 null animals have 20 % higher steady state levels of collagen synthesis the proposition based on increased proline incorporation (Gardner et al. 1999). Skin fibroblasts from scleroderma patients show upregulated collagen type I synthesis with concomitant downregulation of α1 subunit (Ivarsson et al. 1993). In accordance with former observations, an α1 negative osteosarcoma cell line failed to downregulate collagen synthesis in collagen gels while α1 expressing cells were able to do that (Riikonen et al. 1995c). Also α1β1 mediated downregulation of α1(I) mRNA levels both *in vitro* and *in vivo* was detected (Langholz et al. 1995, Riikonen et al. 1995c, and Gardner et al. 1999). Despite the increased collagen type I synthesis, and probably due to increased collagenase

activity, the α1β1 null mice showed no differences in dermal thickness if compared to skin of wild-type animals (Gardner et al. 1999). In the regeneration of experimentally fractured long bone these mice developed significantly less callus tissue than wild-type animals and showed defect in cartilage formation. In the mRNA level reduced synthesis of collagen types II, IX, and X was detected (Ekholm et al. 2002). Also, it was reported that α1 null mice tumor angiogenesis is reduced by over expression on matrix metalloproteinases (MMPs) and consequent generation of angiostatin from circulating plasminogen (Pozzi et al. 2000). The changesin MMP-1, -7, -9 and -13 levels were detected. The increased MMP collagenase activity is presumably the result of a deficiency in the activation of the Ras/Shc/MAPK pathway or increased binding of α2β1 dimer in the absence of α1β1. (Pozzi et al. 1998, Ravanti et al. 1999, Pozzi et al. 2000, and Heino, 2000). In wild-type cells, a subset integrins, including α1β1, have been shown to activate the mitogen activated protein kinase (MAPK) and extracellular signal related kinase (ERK) via recruitment of Sch and activation of Ras (Wary et al. 1996, Mainiero et al. 1997, and Pozzi et al. 1998) suggesting a role in regulation of cell proliferation in three dimensional collagenous environments. This pathway downregulates the type I collagen gene expression as well (Davis et al. 1996).

2.2.3.2 Integrin α2β1 - a primary receptor for collagen type I

Staatz et al. (1989) reported the purified α2β1 integrin containing liposomes adhered to collagen type I, II, III, IV and the adherence was Mg^{2+} ion dependent. Effective liposome adherence to collagen type V was not detected. In contrast to α1β1 dimer, the α2β1 integrin favors typically collagen type I over type IV in binding experiments (Kern et al. 1993, Kern & Marcantonio 1998, and Tulla et al. 2001). The adhesion of NIH-3T3 cells to collagens was found to be mediated by α2β1, but not by α1β1 integrin in Mn^{2+} dependent way. In addition to former positive findings, Saelman et al. (1994) showed α2β1 integrin mediated platelet adhesion to collagen types V, VI, VII, and VIII under physiological divalent cation concentration either under stasis or flow. The function blocking monoclonal antibody (176D7) against α2 subunit completely inhibited platelet adhesion to all collagens tested. Tuckwell et al. (1996) confirmed recombinant α2I domain binding to collagen types I, II, and IV. In their study, the α2 integrin subunit I domain was found to bind specifically also to collagen type XI. Binding to collagen type X was shown by Luckmann et al. 2003. In addition to collagens tested, α2β1 integrin binds other matrix components like laminins and tenascin-1. However, as well as α1β1, the α2β1 integrin recognition is partly cell type dependent, and indicated by the fact that fibroblastic dimers did not recognize these glycoproteins (Elices & Hemler 1989, and Languino et al. 1989, O´Connell et al. 1991, Sriramarao et al. 1993, Colognato et al. 1997, and Ettner et al. 1998). For α2β1 ligands summarized see Table 1.

Two studies with homozygous α2 integrin subunit knock-out mice has been published. Holtkotter et al. (2002) reported that no obvious anatomical or histological were observed in mutant animals if compared to heterozygous and wild-type littermates. Further, knock-out animals are fertile and their fetuses does not show any increased embryonic lethality. These findings are surprising if compared to convincing *in vitro* data suggesting that the tissue morphogenesis could be impaired due to lack of proper adhesion, spreading,

and migration mediated by α2β1. Functional compensation by other collagen or laminin receptor, like α1β1 integrin, might explain this subtle phenotype. As exception in anatomy, Chen et al. (2002) showed with quantitative analysis that mammary gland branching complexity is markedly diminished in the α2 subunit deficient animals. In this study gross and histological evaluation was carried out also for heart, lungs, kidneys, gastrointestinal tract, pancreas, skin and reproductive tracts revealing no abnormalities. Interestingly, the aggregation of α2 subunit deficient platelets to fibrillar collagen type I was delayed with prolonged lag time especially at low collagen concentrations but not reduced (Holtkotter et al. 2002). A similar delay in collagen-induced aggregation has been observed on human platelets in the presence of α2β1 dimer function blocking antibodies (Coller et al. 1989), in integrin β1 subunit deficient mouse platelets (Nieswandt et al. 2001), or in mouse platelets lacking GPV (Moog et al. 2001). Importantly concerning hemostasis, α2 subunit is neither essential for megakaryocyte development or platelet production. However, α2 subunit's absence significantly alters the expression levels of other α subunits within β1 family leading for different ratios of αβ1 combinations (Holtkotter et al. 2002). These findings indicate rather a supportive than an essential role of α2β1 integrin in platelet-collagen interactions (Holtkotter et al. 2002, and Chen et al. 2002). Possibly α2β1 integrins are not essential for development but may be needed for tissue repair, host defense, or other challenges that the adult organism has to meet. The defects stated most likely reflect a reduced stability of the initial platelet-collagen interaction due to the lack of collagen binding sites on the cells (discussed in Holtkotter et al. 2002). In agreement with this is the unexpected observation that knock out animals do suffer bleeding anomalies (Holtkotter et al. 2002, and Chen et al. 2002).

The studies with wild-type cells in three-dimensional collagen type I matrices have shown that the ligand binding of α2β1 triggers MMP-1 (Langholz et al. 1995, and Riikonen et al. 1995c) and MMP- 13 expression (Ravanti et al. 1999) and induces collagen gel contraction (Chan et al. 1992, Langholz et al. 1995, and Riikonen et al. 1995a). In addition to matrix remodeling functions and in contrast to α1β1, the induced collagen type I synthesis was detected (Ivaska et al. 1999b, and Riikonen et al. 1995c). The role of tyrosine-specific protein kinases in these processes, as well as an involvement of protein kinase C (PKC) was proposed. Increased levels of α2 and MMP-1 mRNA in collagen gel-stimulated skin fibroblasts were abrogated by chemical inhibitors for PKC-zeta, and thus, a three-dimensional collagen lattice seems to maintain the dermal fibroblast phenotype, at least in part, through the activation of this PKC isoform (Xu & Clark 1997) even though the evidence is indirect. Also, the effects of nuclear factor kappa-B was detected in this study. Ravanti et al. (1999) showed in kinase assays that collagen type I dependent induction of MMP-13 in dermal fibroblasts requires p38α mitogen-activated protein kinase activity, and is inhibited by activation of ERK-1 and -2. In cells expressing the α2β1 integrin this stress and cytokine-related p38α activity stayed up for several days while it was downregulated in cells expressing chimeric α2 subunit with α1 integrin cytoplasmic domain (Ivaska et al. 1995). In contrast to MMP-1, this induction is independent from PKC activity. In the osteogenic cell lines transforming growth factor β (TGF-β) increased the expression of α2β1 integrin and though regulating the collagen gel contraction that experimentally mimics the reorganization of collagenous matrix during development and tissue healing (Heino et al. 1989, Riikonen et al. 1995a, and Ivaska et al. 1999b). The platelet-

derived growth factor (PDGF) stimulated fibroblasts to move over collagen and contract three-dimensional collagen networks in α2β1 integrin dependent way (Xu et a., 1996). The over expression of α2β1 in osteosarcoma cells prevents α1β1 mediated downregulation of collagen type I synthesis by enhancing upregulation of collagen mRNA levels (Riikonen et al. 1995c). The increase in collagen type I synthesis in α2 transfected human osteosarcoma cells (Saos-2) lacking endogenous α2β1 was shown to be dependent on p38α activity. Based on studies with inhibitors and dominant negative mutants of various signaling proteins, like Rho-family GTPase Cdc42 and dual protein kinases MEK-3 and -4 were found to be essential for this response mediated by intracellular domain of α2β1. On the other hand, the α2β1 integrin is unable to induce ERK activation (Ivaska et al. 1999b).

2.2.3.3 Integrins α10β1 and α11β1 - the novel family members

The α10β1 integrin (Camper et al. 1998) and α11β1 (Velling et al. 1999) are novel collagen binding integrins evolved to separate evolutionary branch parallel to the brace of α1 and α2 subunits (Velling et al. 1999). Little is known so far with respect to newcomers' biology. The α10β1 integrin was identified from human and bovine chondrocytes and human chondrosarcomas by affinity purification followed by immunoprecipitation (Camper et al. 1998). In addition it is expressed at the mRNA level in adult non-chondrogenic tissues like brain, spleen, small intestine, liver, lung, testis, ovary, salivary, adrenal, and glands, pancreas, prostate, skin, bone marrow, muscle, heart, and in fetal brain and liver (Lehnert et al. 1999a). According to amino acid sequence analysis the α10 integrin subunit share the general structure of integrin αsubunits. The large extracellular N-terminal part (1098 aa) of α10 contains a 7-fold repeated sequence which is predicted to fold to β-propeller like structure. It has further three putative, conserved divalent cation binding sites, a single spanning transmembrane stretch (25 aa), and a short cytoplasmic domain (22 aa). It shows 37 and 35 % overall identity to α1 and α2 subunit structures, respectively. For αI structures in details see following chapters. As well as classical collagen binding integrins it contains I domain formed about of 200 amino acids. Since α10β1 was isolated with collagen type II-sepharose, it is in the first place known to bind collagen type II and also interaction with collagen type I was shown (Camper et al. 1998). Tulla et al. (2001) reported recombinant α10I domain binding to collagen types I-VI and laminin-1 in a Mg^{2+} dependent manner. Binding to α2β1 ligand, tenascin, was only marginal. Further in this study, the α10I binding profile resembled α1I domain by especially intensive binding to collagen types IV ($K_d \approx 300 \pm 100$ nM) and VI ($K_d \approx 350 \pm 200$ nM). However, in contrast to α1I domain, α10I showed about equal binding to collagen type I ($K_d \approx 350 \pm 150$ nM). Also interaction with collagen type IX was observed recently (unpublished data from this project). The binding of to ligands was clearly mediated by α10I domain as indicated in Table 1.

The α11β1 integrin was detected in first place from fetal muscle cells (Gullberg et al. 1995, and Velling et al. 1999). It is shown to be expressed at the mRNA level in various adult tissues: uterus, heart, colon, lung, brain salivary and thyroid glands, muscle, testis, prostate, small intestine, bladder, and stomach. (Velling et al. 1999, and Lehnert et al. 1999b). In addition it has been localized to human osteoblast cell lines (Lehnert et al. 1999b), mesenchymal

cells in cartilage of developing skeleton, mesenchymal cells in invertebral discs and keratinocytes of cornea (Tiger et al. 2001). The deduced α11 protein shows the typical structural features of integrin α subunits and is similar to a distinct group of α subunits from other collagen-binding integrins. Amino acid sequence comparisons reveal the highest identity of 42 % with the α10 integrin subunit. The α11 cDNA encodes a mature protein with a large 1120-residue extracellular domain that contains an αI domain of 207 residues and is linked by a transmembrane domain to a short cytoplasmic domain of 24 amino acids (Velling et al. 1999). In adhesion studies, the α11β1 mediated cell attachment to collagens I and IV (with a preference for collagen I) and formed focal contacts on collagens. In addition, α11β1 mediated contraction of fibrillar collagen gels in a manner similar to α2β1, and supported migration on collagen type I in response to chemotactic stimuli (Tiger et al. 2001). Zhang et al. (2003) showed recently that binding to collagen types I, II, and VI was mediated by α11I domain.

TABLE 1 The αI domain containing integrins and their known ligands. The known integrin αI domain involvement in ligand binding is indicated (+). For αI domain see chapters 2.2.4.1 and 2.2.4.1.1.

Ligand for α1β1 integrin	**α1I involvement in ligand binding**	**References**
collagen type I	+	Kern et al. 1993 Kern et al. 1994 Riikonen et al. 1995b Calderwood et al. 1997 Tulla et al. 2001
collagen type II	not shown	Loeser et al. 2000
collagen type III	not shown	Loeser et al. 2000
collagen type IV	+	Kern et al. 1993 Kern et al. 1994 Riikonen et al. 1995b Calderwood et al. 1997 Tulla et al. 2001
collagen type V	+	Riikonen et al. 1995b Ruggiero et al. 1996
collagen type VI	+	Loeser et al. 2000 Tulla el al., 2001
laminin-1	+	Kern et al. 1994 Calderwood et al. 1997
laminin-2	+	Kern et al. 1994 Calderwood et al. 1997
matrilin-1	not shown	Makihara et al. 1999

Continues.

Continues.

C-propeptide of col I	not shown	Davies et al. 1997

Ligand for α2β1 integrin	**α2I involvement in ligand binding**	**References**
collagen type I	+	Staatz et al. 1989 Kern et al. 1993 Kern et al. 1994 Riikonen et al. 1995b Tuckwell et al. 1996 Calderwood et al. 1997 Tulla et al. 2001
collagen type II	+	Staatz et al. 1989 Tuckwell et al. 1996
collagen type III	not shown	Staatz et al. 1989
collagen type IV	+	Staatz et al. 1989 Kern et al. 1993 Kern et al. 1994 Riikonen et al. 1995b Tuckwell et al. 1996 Calderwood et al. 1997 Tulla et al. 2001
collagen type V	not shown	Saelman et al. 1994
collagen type VI	+	Saelman et al. 1994 Tulla et al. 2001
collagen type VII	not shown	Saelman et al. 1994
collagen type X	not shown	Luckman et al. 2003
collagen type XI	+	Tuckwell et al. 1996
laminin-1	not shown	Ettner et al. 1998
laminin-2	not shown	Colognato et al. 1997
tenascin-1	not shown	Sriramarao et al. 1993
decorin	not shown	Guidetti et al. 2002
jararhagin	not shown	DeLuca et al. 1995
jaracetin	not shown	DeLuca et al. 1995
chondroadherin	not shown	Camper et al. 1997
C-propeptide of col I	not shown	Weston et al. 1994 Davies et al. 1997
echovirus-1	+	Bergelson et al. 1993

Ligand for α10β1 integrin	**α10I involvement in ligand binding**	**References**
collagen type I	+	Tulla et al. 2001
collagen type II	+	Camper et al. 1998 Tulla et al. 2001

Continues.

Continues.

collagen type III	+	Tulla et al. 2001
collagen type IV	+	Tulla et al. 2001
collagen type V	+	Tulla et al. 2001
collagen type VI	+	Tulla et al. 2001
collagen type IX	+	Unpublished data from this project
laminin-1	+	Tulla et al. 2001

Ligand for α11β1 integrin	**α11I involvement in ligand binding**	**References**
collagen type I	+	Zhang et al. 2003 Tiger et al. 2001
collagen type II	+	Zhang et al. 2003
collagen type IV	not shown	Tiger et al. 2001
collagen type VI	+	Zhang et al. 2003

Ligand for αLβ2 integrin	**αLI involvement in ligand binding**	**References**
ICAM-1	+	Landis et al. 1993
ICAM-2	not shown	Staunton et al. 1989
ICAM-3	+	Landis et al. 1993

Ligand for αXβ2 integrin	**αXI involvement in ligand binding**	**References**
fibrinogen	not shown	Loike et al. 1991
iCBb	+	Malhotra et al. 1986 Bilsland et al. 1994
ICAM-1	+	Diamond et al. 1993

Ligand for αDβ2 integrin	**αDI involvement in ligand binding**	**References**
ICAM-3	not shown	Van der Vieren et al. 1995
VCAM-1	+	Van der Vieren et al. 1999

Ligand for αMβ2 integrin	**αMI involvement in ligand binding**	**References**
ICAM-1	not shown	Van der Vieren et al. 1995
ICAM-3	+	Diamond et al. 1993
fibrinogen	+	Diamond et al. 1993 Walzog et al. 1995
iC3b	+	Diamond et al. 1993

Continues.

Continues.

Factor X	not shown	Altieri & Edgington, 1988
NIF	+	Moyle et al. 1994 Rieu et al. 1994
heparin	+	Diamond et al. 1995
kininogen	not shown	Gustafson et al. 1989
compl. factor H	not shown	DiScipio et al. 1998
GPIb	+	Simon et al. 2000a
uPAR	+	Simon et al. 2000b
E-selectin	not shown	Kotovuori et al. 1993
Cyr61	+	Schober et al. 2002
CTGF	+	Schober et al. 2002
Fibronecti	not shown	Thompson & Matsushima, 1992
thrombospondin	not shown	Nathan et al. 1989
laminin	not shown	Nathan et al. 1989 Thompson & Matsushima, 1992 Walzog et al. 1995
collagen type I	not shown	Monboisse et al. 1991
collagen type II	not shown	Walzog et al. 1995
collagen type VI	not shown	Walzog et al. 1995
Candida albicans	not shown	Forsyth & Matthews, 1996
Leishmania gp63	not shown	Russell & Wright, 1988
Bordetella pertussis	not shown	Relman et al. 1990
hemocyanin	not shown	Shappel et al. 1990
elastase	not shown	Cai et al. 1996
myeloperoxidase	not shown	Johansson et al. 1997
catalase transferrin	not shown	Davis, 1992
casein	not shown	Davis, 1992
trypsin inhibitor	not shown	Davis, 1992
BSA	not shown	Frieser et al. 1996
ovalbumin	not shown	Davis, 1992
non-protein ligands	not shown	For details and references see Yakubenko et al. 2002

Ligand for αEβ4 integrin	**αEI involvement in ligand binding**	**References**
not known ligands		For characterization see Kajiji et al. 1989

Ligand for αEβ7 integrin	**αEI involvement in ligand binding**	**References**
ICAM-1	not shown	Cresswell et al. 2002

Continues.

Continues.

E-cahderin	not shown	Cresswell et al. 2002

2.2.4 Structure of integrins - a handshake between the subunits

The Fig. 3 presents schematically the organization of the different domains found in α2β1 integrin dimer. Both integrin glycoprotein subunits are type I transmembrane proteins. They contain typically a large extracellular (≈ 600-1100 aa) and a relatively short cytoplasmic (≈ 30-50 aa) domain lacking the enzymatic activity connected by short and single, hydrophobic transmembrane stretch (≈ 20-30 aa). However, as exception to shortness of cytoplasmic part, the β4 subunit has an intracellular domain made of over 1000 amino acid residues. The non-covalent contacts between subunits involve primary their NH_2-terminal halves. In α subunit this area contains, the β propeller with or without αI domain and in β subunit the βI like domain and so called hybrid domain (Humpries 2000). By electron microscope, typically a globular head (≈ 8 x 12 nm) with two rod-like tails (≈ 14-18 nm) can be observed as result of dimerization to functional receptor. For example of microscopical studies see Carrell et al. 1985, Nermut et al. 1988 and Weisel et al. 1992. As well, in X-ray crystallography, an ovoid αVβ3 dimer "head" and two "tails" was observed (Xiong et al. 2001). According to the former, the α subunit tail contains three large b sandwich domains. Near the plasma membrane are situated two sequential "calf" domains that are further linked via a highly flexible "knee" to "thigh" domain neighboring the globular head. Correspondingly, the β subunit tail contains, a cystatin-like fold, four successive epidermal growth factor (EGF)-like repeats, a PSI domain (standing for plexins, semaphorins, and integrins), and an Ig-like "hybrid" domain nearest to the βI like domain. The PSI domain is linked to hybrid domain and further to the linker between 4th EGF-repeat and the hybrid domain. The biology of extracellular domains, i.e. ligand binding area of integrin α and β subunits, is reviewed in the following.

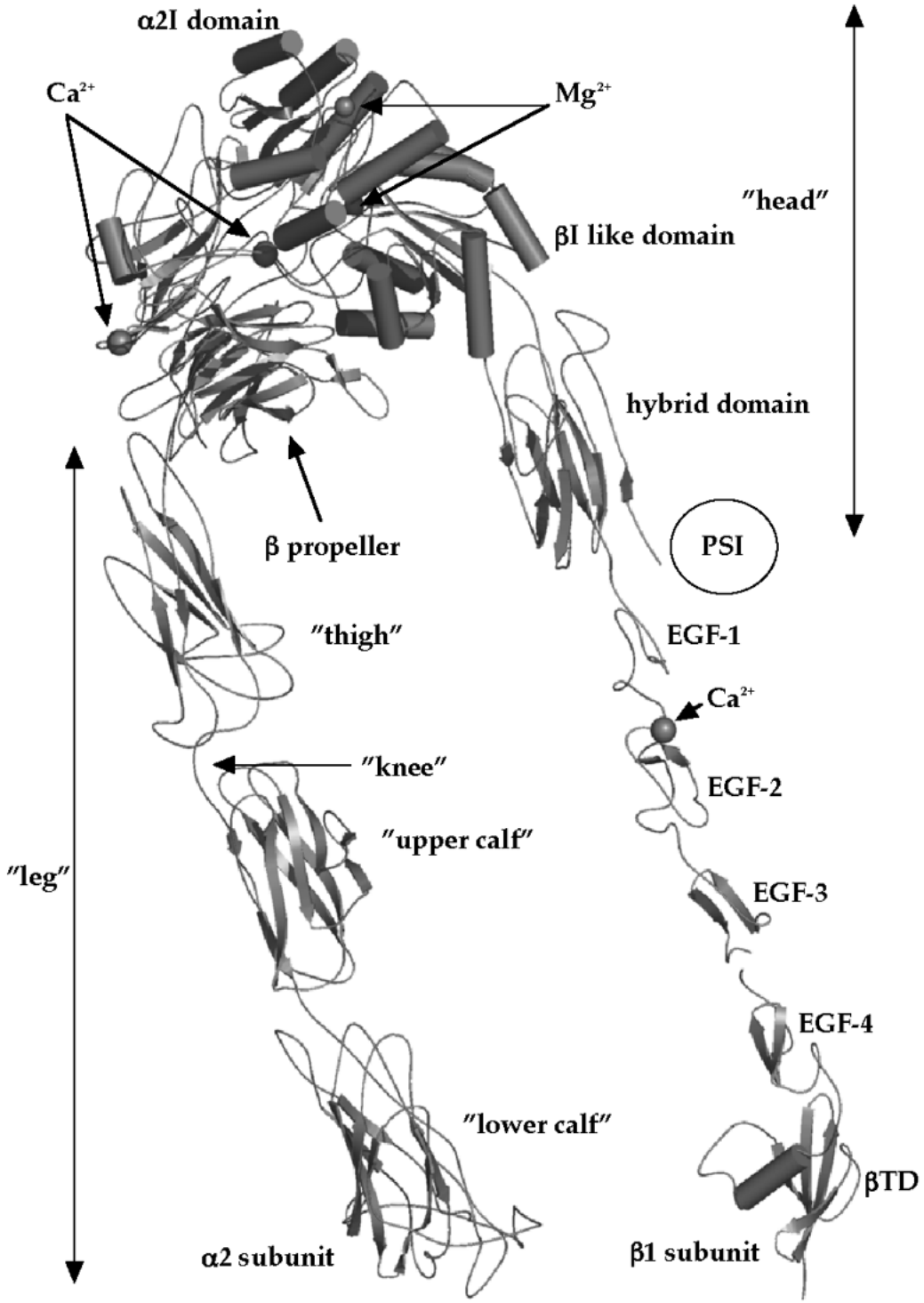


FIGURE 3 Molecular model of α2β1 integrin extracellular domain in active conformation. The C-terminal αI domain and βI like domain push the hybrid domain into active/open conformation with the leg regions separated. PSI domain is not modeled but is present close to the hybrid/EGF- 1 interface as marked. Divalent metals in MIDAS sites, i.e. Mg^{2+}, are indicated by smaller spheres. Larger spheres indicate Ca^{2+} ions. Model is modified with permission from presentation by White et al. (2003).

2.2.4.1 Integrin α subunit - a ground for collagen binding

Integrin α subunits share an overall identity of 20 - 40 % (Hynes 1992). The conserved NH_2-terminal segment (≈ 440 aa) of integrin α subunit, i.e. ligand binding area, contains seven homologous sequence repeats (≈ 60 aa) forming the structure called β propeller (Corbi et al. 1987, and Springer 1997). The propeller like conformation was supposed in the first place based on molecular modeling (Springer 1997). In this structure, the repeats contain FG (phenyl-alanyl-glycyl) and GAP (glycyl-alanyl-prolyl) consensus sequences and are therefore generally termed as FG-GAP repeats. Each FG-GAP repeat is folded to four stranded β sheets (strands A-D) and these triangle shaped structures are arranged in a torus around a pseudosymmetry axis. Analogous toroidal propeller like conformation is found in many other proteins, including the β subunits of G-proteins. Theoretical proposal concerning β propeller in integrins was later approved by Xiong et al. (2001) based on crystallographic data from extracellular portion of integrin αVβ3. According to this, the inner strand (strand A) of each repeat, "a sharp plade of triangle", lines the predominantly amide and carbonyl oxygen covered by a channel at the center of the propeller. The strand D lines externally the propeller edge. Importantly concerning crystal, the structure was determined in the presence of Ca^{2+} ion. Putative Ca^{2+} binding motifs (DxD/NxD/NxxxD), called EF-hands, are present from the 4th to 7th repeat in non αI domain containing integrins and in repeats 5 through 7 in αI domain containing integrins. The solvent exposed Ca^{2+} binding sites are situated in the β hairpin loop between strands A and B at the bottom of propeller. However, these bivalent cation binding motifs in integrins differ from "classical" EF-hands sequences by lacking co-ordination residues at position 12.

One hypothesis about integrin-ligand binding suggests that recognition site in ligand might bind at or near to the EF-hand-like sequences and take place of the missing residue and co-ordinate directly the bound cation (Tuckwell et al. 1992). However, later it was shown that cation binding sites does not determine the specificity of ligand recognition by having probably instead a more regulatory role (Mould et al. 2000). This study compared α5β1 and αVβ1 integrins and their chimeric based changes in ligand specificity, and it is still possible that they do have similar ligand recognition mechanism though the importance of cation binding residues was not seen. However, as generally accepted, the ligand binding sites in integrins are mapped to the FG-GAP repeat area in the upper surface of β propeller (Ginsberg 1995 and Loftus et al. 1994). More closely, the putative ligand binding area in non αI domain containing integrins is supposed to be located in loop like structure between adjacent lying repeats 2 and 3 (Springer, 1997, and Mold et al. 2000). According to Mold et al. (2000), the first three NH_2-terminal repeats contain the epitopes of function blocking monoclonal antibodies against non αI domain containing α5 integrin subunit. Further, the amino acid sequence Ala107-Trp226, corresponding approximately to second and third repeats, was shown to be crucial for ligand binding. In αI domain containing integrins, this additional loop like (≈ 200 aa) structure is situated between the second and third FG-GAP repeat.

2.2.4.1.1 The αI domain structure

Nine of known integrin α subunits have αI domain, the letter "I" standing for both "inserted" and "interactive" forming a monophyletic group separate from other integrins (Hughes 2001). These structures are homologous to modular multidomain A1 and A3 structures in von Willebrand factors (vWF) found in one or more copies in great variety of cell adhesion supporting and mediating molecules and therefore also term "A domain" is used in context of integrins by some research groups. In evolution, the αI domain containing integrins seem to be a *chordate*-specific radiation of the gene family because they have been found only in vertebrates and in the primitive *chordates,* but are absent from *C. elegans* and *D. melanogaster* (Hynes & Zhao 2000 and Whittaker & Hynes 2002). In the integrin family, five of αI domain containing subunits (αM, αX, αD, αL and αE) as separate cluster are expressed solely on leukocytes where they mediate first of all cell-cell interactions. Further, the αI domain containing subunits as separate cluster, i.e. subunits α1 (accession code X68742), α2 (X17033), α10 (AF112345), and α11 (AF137378) dimerize with β1 subunit (X68969) and mediate collagen binding as discussed before (Hughes, 2002 and Whittager & Hynes 2002). The primary ligand recognition and binding of all αI domain containing integrins seem to be mainly mediated through their αI domain (for example see Calderwood et al. 1997, Champe 1995, Huang & Springer 1995, Kamata & Takada 1994, and Tuckwell et al. 1995). The recombinant αI domains are independently folding structures, and they retain many of functional properties of the parent integrin, and therefore used generally as models for studying integrin functions (for example see Muchowski et al. 1994 and Tuckwell et al. 1995).

Mediating αI structures from cell-cell interactions have been solved for αMI (Lee et al. 1995a, Lee et al. 1995, and Baldwin et al. 1998) and αLI (Qu & Leahy 1995, and 1996) domains with and without metal ions. Within the collagen binding integrin family, the human α2I domain was the first crystal structure solved by Emsley et al. (1997) at 1.9 Å resolution. The rat recombinant α1I domain structure at 2.2 Å followed shortly afterwards (Nolte et al. 1999). This structural data was further supplemented by Rich et al. (1999) and Salminen et al. (1999). Presentations concerning α1I and α2I domains, showing sequence identity of about 50 %, are based on publications stated. In addition, some unpublished data from this project is presented in the text. As expected according to sequence alignment of αM (structural similarity 26.7 % to α2I; Emsley et al. 1997), αL subunit (structural similarity 24.0 % to α2I; Emsley et al. 1997), and αI domain crystal structures, the α1I and α2I domains adopt the classic dinucleotide-binding fold, called alpha/beta Rossmann fold present in many intracellular enzymes. It contains seven amphipathic α helices (termed α1-α7) surrounding a highly conserved core of five parallel, hydrophobic b sheets or strands (termed βA-βE) and one short antiparallel β sheet (termed βF). Different to vWF A domains, integrin αI domains contain a metal ion-dependent adhesion site (MIDAS) with bound ion at the C-terminus of the β sheets. Five α helices (α1, α3, α4, α6, α7) are parallel to one another and antiparallel to neighboring β strand. The α2 helix is parallel to βB strand where its N-terminal end is connected to the C-terminal end of the βA strand through the short anti-parallel βC strand. The overall structure of Rossmann fold is highly conserved among integrin αI domains and vWF A domains with RMS

deviations of 0.6-0.7 Å. Typically, the hairpin formed by βB and βC sheets and protrusion at the end of βC are almost identical in all αI domains. Instead, the α helices are more variable. Only the α1 and α4 helices show similarity in length and orientation within the Rossmann fold family. In α2I and vWF-A3 domains, the helix α2 is replaced by short turn and α3 helix is extended by an additional turn. The C-terminal α7 helix in α2I has similar conformation in αMI and vWF-A3 domain but different to αLI, in which this helix exposes a large hydrophobic crevice (Qu & Leahy 1995). This helix is supposed to be important at least in αMI function by propagating the structural changes from the MIDAS face to the rest of the integrin (Lee et al. 1995b). In α1I and α2I domain MIDAS areas, the phenylalanine at the top of α7 helix in αMI (in position 302) and αLI, is replaced by a glutamic acid in the positions 321 and 318, respectively. In α2I this glutamic acid makes hydrogen bonds with the main chain nitrogen of His288 and the Asp257. This stretching structure turns upwards and creates a cavity which is occupied by a water molecule that is not found in corresponding areas of α1I, αMI and αLI structures. Further, the biggest difference between α1I and α2I domains are shifts in the C-terminus of α7 helix that can reach up to 2 Å in the last few main atoms of the helix.

All known metal ion interacting proteins, with dinucleotide binding fold architecture, have their binding sites in or in the vicinity of MIDAS. Indeed, this site occupied by divalent metal ion plays a major role in ligand binding (for example see Kern et al. 1994, Kamata & Takada 1994, and Tuckwell et al. 1995). This area is composed of loops βA-α1, α3-α4, βD-α5, and βE-α6. All these structures, but βE-α6, have been shown to be involved in ligand binding of αMI and αLI domains (Huang & Springer 1995, Champe et al. 1995, and Rieu et al. 1996). Despite the fact that the main chain conformation is conserved, the loop surfaces have highly variable residue composition within αI domains, therefore being considered to be main determinants of ligand binding specificity. The α2I MIDAS area in loop βA-α1 with 2-residue deletion contains conserved, metal-coordinating $D_{151}x_{152}S_{153}y_{154}S_{155}$ motif. In α1I, the corresponding motif is situated in positions 154-158. The non-conserved residues in α2I are at the x position buried glutamic acid, instead of usual glycine, and in the y position asparagine. The α2Iα3-α4 which wraps over the top of βA and βB sheets has a one residue insertion (R218), which creates space for glutamine152. The βD-α5 loop is similar in α1I, α2I, αMI, and vWF-A3 domains. In addition to conserved DxSyS motif α2I MIDAS area contains accompanying, critical residues for integrin collagen interaction, namely Asp254, and Thr221. In α1I, these conserved residues are situated in positions 257 and 224, respectively. In α2I domain the metal is coordinated by side chains in residues Ser153, Ser155 and Asp254 and three water molecules (w1-w3) with distances of 2.1±0.1 Å, making strong bonds in an octahedral arrangement. In α1I water w3 is buried and bonded to Asp150 (2.4 Å) and Thr220 (2.5 Å). The water w2 is bonded to the ion with an appropriate hydrogen bonding distance to the backbone carbonyl of residue Asp253 (2.9 Å), which leaves the most exposed w1 as the only water in the coordination sphere with only a single bond to the molecule via metal. In addition, important for function, the aspartic acid in α1I position 154 and the aspartic acid in α2I position 151 make hydrogen bonds to serine in positions 156 and 153, respectively (Kamata et al. 1994). The conserved threonine in α2I position 221 seems not to coordinate the metal directly, but makes well a

hydrogen bond to one of water molecules (Mg-OH(Thr)). It has been shown that this residue is the only amino acid in the metal binding area that is absolutely critical for recombinant α2I domain binding to collagen type I (Kamata & Takada 1994). In accordance, Kamata et al. (1995) reported that the threonine residue in position 206 in αM and threonine in position 209 in αL corresponding to Thr221 of α2I are critically involved in the ligand interaction with β2 integrins.

The conformation of α1I and α2I domain as described above is very similar to αMI and αLI MIDAS area when their metal binding site is occupied by Mn^{2+}, defined as the "closed" conformation (Lee et al. 1995a, and 1995b). However, different to "closed" conformation, the αMI crystal containing Mg^{2+} displayed conformation called the "open", or in the first place, analogous to the G proteins, "active" conformation. Indeed, the first αMI crystal with Mg^{2+}, (Lee et al. 1995a, and 1995b) showed significant differences to the following αMI, (Lee et al. 1995a, and Baldwin et al. 1998) and αLI crystals (Qu & Leahy 1995) when the MIDAS was occupied by Mn^{2+}. In the "open" conformation a glutamate provides the sixth metal coordination site. In addition, the difference between two states are dominated by solvent exposed phenylalanins in positions 275 (in middle of βE-α6 loop in αMI) and 302 due in conformational changes to "open" form. In "closed" αMI conformation, the Phe275 occupies hydrophobic pocket in correspondence to Phe299 at α6 helix in α1I domain. There was uncertainty about "activity" between these conformations until the Baldwin et al. (1998) showed that the "open" or "active" conformation proposed by Lee et al, is likely a construct artifact. In addition, Li et al. (1998) presented that crystallized αMI domain can adopt also the "open" conformation despite the presence of Mn^{2+} and this process is independent of the nature of metal.

Despite the overall structural similarity and the conserved protein structures reviewed above, when comparing collagen binding integrin αI domains to cell-cell interactions mediating integrin αI domains, an obvious difference can be seen. In α1I and α2I domain βE-α6 loop, a new turn-and-a-half of a helix, composed of residues of 282-288 (LGSYNRG) and 283-289 (LGYLNRN) was observed, respectively (Emsley et al. 1997, and Nolte et al. 1999). Emsley and co-workers termed this additional main αI domain body protruding loop as αC helix. The αC helix structure is highly similar in all collagen binding integrin αI domains. Within α1I and α2I domains the RMS deviation between helix loops are 0.78 Å and 1.24 Å for equivalent atoms. The α1I His288 is replaced in α2I with Tyr285 with both, however, adopting the same side chain conformation. Additionally, Leu286 in α2I is replaced by Tyr289 that is stabilized by a hydrogen bond with the main chain of Glu259. The αC helix residues and the loop between helices α3 and α4 point toward the ligand binding site and make the crevice of collagen binding integrin αI domains narrower and more convex than cell-cell interactions mediating integrin αI domain areas. The importance of αC helix in ligand binding is under crosstalk and is discussed in this thesis as well.

2.2.4.1.2 Integrin-collagen interaction - a cocrystal

Emsley et al. (2000) determined the crystal structure of α2I domain (resolution 2.1 Å) with bound, collagen mimicking, synthetic GFOGER peptide. As reviewed before this motif in collagen has been shown to be recognized by collagen binding integrins (Knight et al. 1998, Knight et al. 2000, and Xu et al. 2000). Collagen α chain peptides are situated in one residues stagger and coordination of each analogous residue is unique. In this presentation these strands are called strand 1, 2, and 3 as viewed from their N-terminal region. Triple helical peptide used was composed of 21 residues (65 Å x 12 Å) and bound to the upper edge of αI domain, with footprint 25 Å long and 10 Å wide. Three MIDAS loops coordinating the metal ion seem to interact with collagen strands 2 and 3. No interaction between strand 1 and αI domain was detected. The peptide strand 2 arginine (-GFOGER-) forms salt bridges to Asp219 in αI domain loop α3-β4 and Asp215 and carbonyl H-bonds to His258. The strand 2 phenylalanine (-GFOGER-) is situated by van der Waals contacts on the excavation formed by residues Glu215 and Asn154 (from loop βA-α1) side chains from αI domain. Hydroxyproline (-GFOGER-) carbonyl hydrogen bonds well to Asn154. Peptide glutamate (-GFOGER-) bonds to metal in MIDAS and to residue Thr221 in loop α3-β4. The strand 3 three phenylalanine makes hydrophobic van der Waals contacts with Tyr157 (from loop βA-α1) and Leu286, while collagen arginine lies in an acidic pocket in the vicinity to conserved residue Glu256 (from loop βD-α5) with weak acidic interactions. Finally, hydroxyproline bonds to Asn154 main chain.

The structure of unligand and ligand α2I domain in presented in Fig. 4. Structural changes occur in αI domain during ligand binding if compared to unligand domain. Principal changes can be observed in three αI domain regions. The MIDAS loops and helix α1 move due to changes in metal coordination. Further, motion between αC helix and helix α6 opens the collagen binding site. Finally, the binding propagates structural changes of C-terminal α7 helix to the opposite pole of the αI domain. The central β sheet retains stable conformation. During transition from unligand ("closed" conformation) to ligand ("open" conformation) state of αI domain, the metal ion moves 2.6 Å towards the loop stated in order to make a direct bond with Thr221. MIDAS loop βA-α1 follows this movement to maintain direct bond via Ser153 and Ser155. The Asp254 and Gly255 in MIDAS loop βD-α5 move laterally so that they loose the direct conjunction to the metal. As a result of this Glu256 forms a new water-mediated bond to the metal. The rearrangement of loops βA-α1 and βD-α5 trigger the reorganization of C helix and α7 helix. Loop βA-α1 is situated against α7 helix when unligand. After collagen binding βA-α1 and helix α1 assist the 10 Å downward movement of α7 helix. This transition breaks partly the salt bridge between Glu318 from α7 helix and Arg288 from the C helix. In addition, movements of loops βA-α1 and βD-α5 bring the side chains of Tyr157 and His258 3Å closer together in order to fit into grooves of the triple helix. The loop βD-α5 is packed in the vicinity of α6 helix in "open" conformation and therefore forces a rearrangement of the side chain of Leu296, which therefore creates close contact with Leu286 from αC helix. Due to steric changes concerning these leucines and loss of stabilizing Glu318-Arg288 salt bridges, the conformation of αC helix changes. The C helix structure unwinds, while the

connecting loop coils up to form an extra turn at the N terminus of α6 helix. The uncoiling produces a dramatic 180° rotation and a shift of Tyr285. During this, residues hydroxyl oxygen moves by 17 Å from its location above the MIDAS motif to form a hydrogen bond with Ser316 at the top of the repositioned α7 helix. In contrast, Leu286 moves 4 Å towards collagen to make van der Waals contacts with collagen strand 3 Phe, and Arg288 moves 6 Å closer to the MIDAS motif where it forms a water-mediated salt bridge to Asp254.

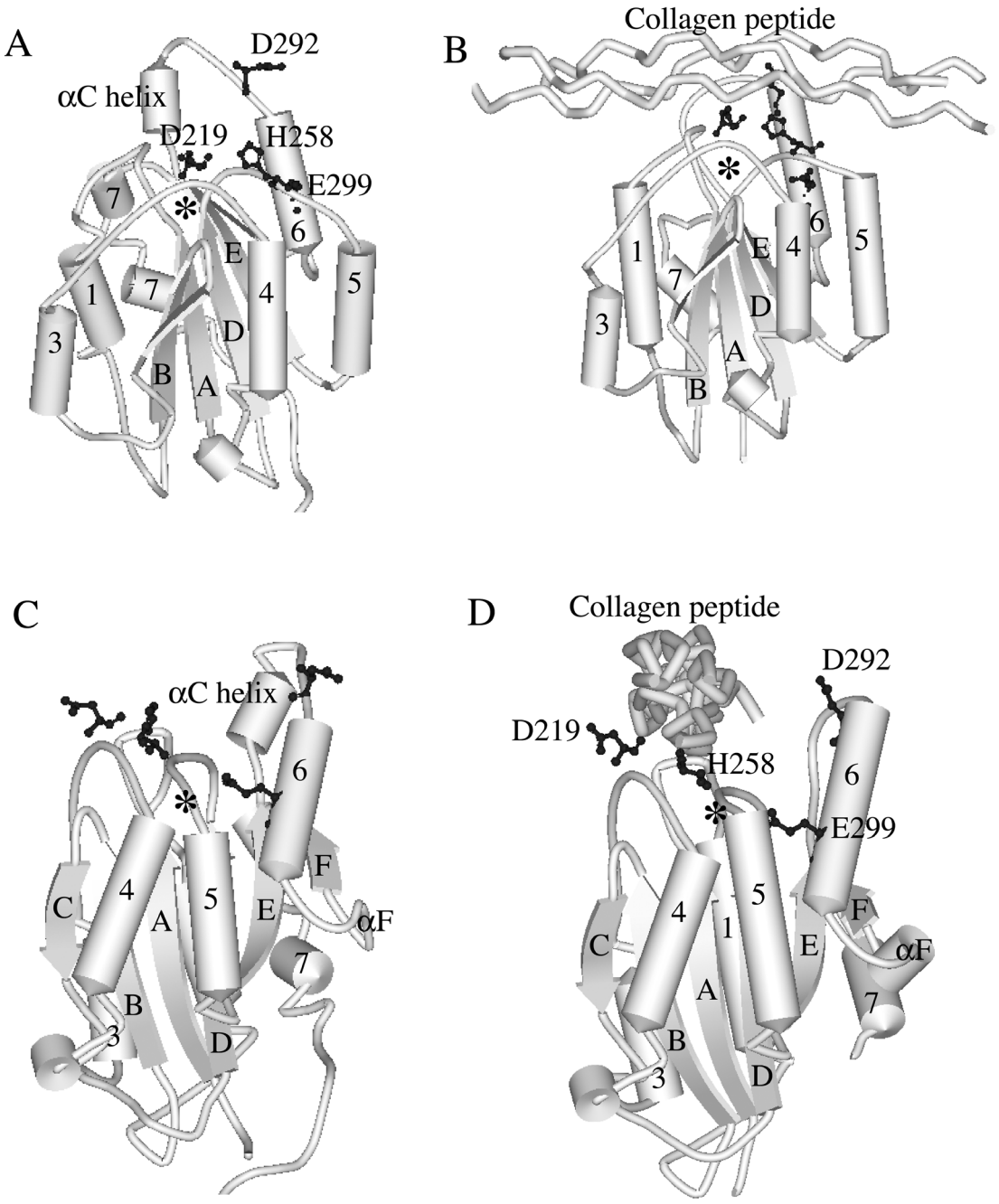


FIGURE 4 Structure of the α2I domain and α2I domain-collagen peptide complex. The α2I domain helices are shown in coverage area as cylinders and named by numerals, however the αC and αF helices are exception. The β strands in coverage area are shown as arrows and named by alphabets. The Mg^{2+} ion situated in MIDAS area is marked with an asterisk (*). Residues studied in this thesis, D219, H258, D292, E299, are marked starting from NH_2-terminus. Panels A and C shows α2I domain without ligand. Panels B and D represents α2I domain occupied by triple helical, GFOGER motif containing collagen peptide. The view is either from the front (A and B) or side (C and D) prorata to αC helix. For conformational changes concerning ligand binding in detail see the chapter above. This illustrated presentation is based on publications by Emsley et al. in 1997 and 2000.

2.2.4.2 Integrin β1 subunit with βI like domain

Ligand recognition by integrins is not limited to their αsubunits. The β1 subunit, found both from cell-cell and cell-matrix interactions mediating integrins, seems to play a central role in regulating integrin dimer affinity and different cellular functions. The monoclonal antibodies (mAbs) against β1 subunit induce comitogenic proliferative signal in CD4 T-lymphocytes on fibronectin (Yamada et al. 1991) and as well as negative signaling effects on T cell proliferation (Groux et al. 1989). In addition some anti-human β1 mAbs inhibit the attachment of human cells to a great variety of ligands. As an example see Freedman et al. 1990, Kramer et al. 1990 Carter et al. 1990 and Mould et al. 1991. Also β1 subunit antibody increase in integrin mediated adhesion is known (Neugebauer & Reichardt 1991, and van de Wiel-van Kemenade et al. 1992). However, most of these studies, as well as following structural studies, are carried out with cell-cell interactions mediating integrin dimers and a minority of them contains β1 subunit as partner of different α sub

The interaction between integrin α and β subunits is mediated via their β propeller and βI like domain (vWFA-like domain), respectively. According to studies with dimerizing αL and β2 subunits, these domains are co-operatively folded to a functional, ligand binding unity when associated (Huang et al. 1997, and Huang & Springer 1997). The I like domain seems to define the part of ligand specificity since the binding is altered when diverse sequences in this area are swapped between subunits (Takagi et al. 1997). The βI like domain area is considered to be especially important in ligand recognition and binding in integrins missing the αI domain.

The βI like domain in the NH_2-terminal part of β subunit is structurally homologous, but not identical to the collagen binding integrin αI domains. This pretense was based in the first place on parallel hydropathical profiles of six integrin αI domains and all eight human integrin β subunits. According to this, the βI like domain is formed by buried hydrophobic β strands and hydrophilic α helices that surround the β sheet (Lee et al. 1995a). Later this structural prediction was supplemented with computer based sequence analysis by Tuckwell and Humpries (1997). According to the former, βI like domain with alternating β strands and α helices situated at a position between 161 and 408 giving a putative domain of 249 amino acids, compared favorably with the ≈ 200 amino acids seen for αI domains. In light of consensus tertiary structure prediction, the secondary structure stated adopts a Rossman fold similar to αI domain. Other models tested, either ignored the α helix content (e.g. β sandwich) or presented alternative ways of organizing the secondary structure (e.g. TIM barrel). Different to the αI domain, the βI like domain shows two insertions, one between β strands B and C, and one between β strand D and α5 helix. The first of these insertions lying near the extremity of the domain has little or no predicted structure, but appears to be supported by a disulphide bond (Calvete et al. 1991). The second loop represents a major departure from the domain, as it lies near the centre of the domain, projecting out from it. Secondary structure prediction indicates that this region contains some β strands, perhaps forming a small sheet. This area seems to be stabilized by a disulphide bond to α4 helix (Tuckwell & Humpries, 1997).

As well, the βI like domain area contains the metal-ion-dependent adhesion site (MIDAS) with highly conserved DxSxS motif (Lee et al. 1995a). In

β3 subunit this site is situated in positions 119-123 and substitution mutations in this area abolish fibrinogen and RGD-peptide binding to αIIbβ3 dimer (Bajt & Loftus 1994). Similarly, a mutation in β1 at position 130 (equivalent to 119 in β3) abolishes fibronectin and invasion binding to αVβ1 (Takada et al. 1992). Significantly, RGD-containing peptides cross-link next to MIDAS in β3 position Lys125. However, in βI like domain there is an additional metal binding site called ADMIDAS occupied by Ca^{2+}. This structure was suggested to be involved in ligand binding based as well on crystal structure of the extracellular portion of integrin αVβ3 (Xiong et al. 2001).

In addition, other MIDAS-related motifs important for function of βI like domain have been found. Takada & Puzon (1993) identified a small region in β1 subunit, namely residues 207-218 (NKGEVFNELVGK), that is critical for the binding of both activating and inhibiting monoclonal antibodies. Similarly, measurements with interspecies chimeric subunits and site-directed mutagenesis were carried out. A conserved epitope nearly in all β subunits is located between two conserved sequences in positions 120-182 and 220-231, which correspond to the two putative ligand binding regions of β3 subunit (Takada & Puzon, 1993, Shih et al. 1993, and Steiner et al. 1993). The cation binding to this area in αIIbβ3 integrin was demonstrated by terbium luminescence and mass spectroscopy (D´Souza et al. 1994). The RGD-containing peptides seem to cross-link to a region containing residues 109-171 of β3 subunit in αIIbβ3 (i.e. corresponding to 120-182 in β1) and to an overlapping region (residues 61-203) in the β3 of αVβ3 (D´Souza et al. 1988, and Smith & Cheresh 1988). Another region of β3 residues 211-222, corresponding to 220-231 in β1, has also been shown to be involved in the interaction with a ligand. This motif mimicking synthetic peptides and antibodies against peptides inhibit competitively fibrinogen binding to αIIbβ3 (Charo et al. 1991). Also arginine substitution to glutamine or tryptophan in β3 position 214 blocks the fibrinogen binding and destabilizes the association of αIIb subunit with β3 in thrombobasthenic patients suggesting that this region is also involved in ligand binding and/or αβ association (Bajt et al. 1992, and Lanza et al. 1992).

3 AIMS OF THE STUDY

Integrins are a large family of heterodimeric cell adhesion receptors involved in cell–extracellular matrix interactions. As important part of this, cells can express collagen binding integrins, namely α1β1, α2β1, α10β1, and α11β1 dimers concomitantly on their surface. The biological functions, such as signal transduction, of these collagen receptors seem to be different.

In mammals there are almost 30 different collagen types and they include among other following subgroups studied in this thesis: fibrillar collagens (like types I, II, III, V), network forming collagens (like type IV), and transmembrane collagens (like types XIII and XVII).

The integrin type cellular receptors for many collagen subtypes are unknown. An interesting possibility is that some collagen subtypes might be selectively recognized by only one collagen receptor. The ligand specifity of classical collagen binding integrins, α1β1 and α2β1, in relation to collagen types stated, was studied.

The specific aims of this doctoral thesis were:

-To clarify if integrin αI domains involment in binding of collagen types I, II, III, and V are in accordance with cellular spreading behavior and related to known integrin binding sequences (I).

-To clarify whether the collagen binding α2β1 integrin have different molecular mechanisms involved in the recognition and binding of fibrillar collagen type I and network like structures forming collagen type IV (II).

-To clarify if collagen binding α1β1, and α2β1 integrins are able to recognize type I collagen in fibrillar form (III).

- To clarify which integrins are receptors for transmembrane collagen types XIII and XVII (I, IV).

4 MATERIALS AND METHODS

4.1 Preparation of collagens

4.1.1 Generation of recombinant ectodomain of human collagen type XIII (I)

Generation of recombinant ectodomain of collagen type XIII was carried out as follows by Hong Ming Tu from Taina Pihlajaniemi´s group (University of Oulu). For N-terminally truncated collagen type XIII expression in insect cells, the construction of recombinant baculoviruses were carried out as described by Snellman et al. (2000a). This truncated form lacking the cytosolic domain is known to form triple-helical molecules normally, but is instead expressed in higher amounts and shed significantly more efficiently from insect cells if compared full-length collagen type XIII (Snellman et al. 2000b). The partial cDNA was generated by PCR using full length human collagen type XIII cDNA as template. The partial cDNA fragment was ligated to full length digest resulting in a construct called del1-38/BlueSrcipt. The del1-38/BlueSrcipt and pVL1392 vector was digested and free insert was ligated to the vector. The resulting construct was co-transfected with modified *Autographa californica* nuclear polyhedrosis virus DNA into *Spodoptera frugiperda* Sf9 insect cells using the BaculoGold transfection Kit (Pharmingen) according to manufacturer's instructions. The High Five insect cells were infected with del1-38 virus and with the 4PHαβvirus coding α and β subunits of prolyl 4-hydroxylase (Lamberg et al. 1996). The supernatant was harvested 72 h post infection and loaded to RESOURSE S column. The preparation was eluted with a linear NaCl gradient and analyzed by non-reduced SDS-PAGE and Western Blotting using an antiserum against the NC3 domain of collagen type XIII (Hägg et al. 1998). The recombinant protein generated corresponded for the amino acids 650-1012 of mature collagen type XIII ectodomain. Before use, the preparations was diluted in PBS and used as such in experiments. For these see chapters 4.2.5. and 4.3.3.

4.1.2 Generation of recombinant human COL15 domain of collagen type XVII (IV)

Generation of recombinant COL15 domain was carried out as follows by Kaisa Tasanen from Leena Bruckner-Tuderman´s group (University of Oulu). The procedure was described previously by Tasanen et al. (2000). For this, a cDNA fragment corresponding to the COL15 domain of human collagen type XVII was amplified by RT-PCR using cultured human primary dermal keratinocyte mRNA as template. The fragment was ligated into a purified episomal expressing vector pCEP-Pu (Kohfelt et al. 1997). Human embryonic kidney 293-EBNA cells were transfected with pCEP-COL15, using the calcium phosphate based method. The transfected cells were grown to confluency and switched to serum-free medium supplemented with ascorbic acid. The media were collected every 48 h, dialyzed and chromatographed on a DEAE-cellulose column. The rCOL15 did not bind to DEAE-cellulose, but a significant amount of contaminating proteins was removed with this purification step. The recombinant protein generated corresponded for the amino acids 567-807 of mature BP180 protein. In one set of experiments the triple helical structure of rCOL15 was denaturated by heating. After dilutions in PBS both native and denatured preparations were used as such in experiments. For these see chapters 4.2.6., 4.3.3., and 4.3.5.

4.1.3 *In vitro* collagen type I fibrillogenesis (III)

The collagen type I stock solution was diluted on ice to final concentrations and the pH of dilutions was adjusted to 7.4. To initiate the fibril assembly solutions were transferred to a spectrophotometer connected to a water bath at 37°C. Control reactions were kept on ice to maintain the monomeric state of collagen. In one set of experiments, the preparation was warmed before pH adjustment. For real time kinetics, the turbidity of the initiated samples as absorbance at 313 nm was recorded for 60 min. At the 60 min time point, the preparations with fibrillar and monomeric collagen (0.5 mg/ml) were used as such in experiments. For these see chapters 4.2.6, 4.2.7., and 4.3.3.

4.2 Studies on integrin αI domain-collagen interactions (I-IV)

4.2.1 Generation of human recombinant integrin α1I and α2I domains (I-IV)

The cDNAs encoding for α1I and α2I domains were generated by PCR as described earlier by Ivaska et al. (1999), by using human integrin α1 and α2 subunit cDNAs as templates. Integrin α1 cDNA (Briesewitz et al. 1993) was a kind gift from Dr. E. Marcantonio (Columbia University) and α2 cDNA (Takada & Hemler, 1989) from Dr. M. Hemler (Dana-Farber Cancer Research Center). In addition in publication II, the shorter wild-type version of α2I domain called ΔC140 was generated, presenting the amino-terminal deletion at the position of the first cysteine140. The PCR-products, pGEX-4T-3 -vector (Pharmacia) for α1I domain and pGEX-2T -vector for α2I domain were ligated. One difference in insert sequence was found in the α1I domain area if compared to sequence

(X68742) originally reported by Briezewitz et al. (1993). Due to this substitution difference in the nucleotide sequence, the lysine will be replaced to glutamate at amino acid position 170 numbered from the start of the mature peptide. This same difference has been reported by Calderwood et al. (1997). They reported also isoleusine instead of threonine at position 228, but we did not find this replacement. Here, the sequence was further checked from another independent source. The predicted amino acid sequence had glutamate at position 170 and isoleusine at position 228. The pα1I and pα2I plasmids were transformed into competent *E. coli* BL 21 cell strain for production of human α1I and α2I as recombinant proteins. The cells were grown under *lac*operon inducer, IPTG. Cells were lyzed and the glutathione sepharose to harvest fusion proteins was added to the lysates. Matrix with bound fusion proteins were transferred onto the chromatography columns and washed to remove non-bound contaminating proteins. In publications III and IV the immobilized GST fusion proteins were competitively eluted from matrix by incubating with glutathione. The SDS and native polyacrylamide gel electrophoresis without, and when necessary with dithiothreitol, was done for both α1I-GST- and α2I-GST-proteins. They used as such for binding experiments. For these experiments see chapter 4.2.6. In publications I and II, the recombinant proteins were cleaved with protease thrombin to separate αI domains from tagged GST-fusion partner. The recombinant α1I domain produced was 227 amino acids and α2I domain 223 amino acids long. They correspond to α1 integrin subunit sequence 123-338 (VSPT...LEATA) and α2 integrin subunit sequence 124-339 (PDFQ...IEGTV). Additionally, both thrombin cleaved recombinant proteins in publications I and II had two non-integrin amino acids at the amino terminal region (GS). In publications III and IV the non-cleaved proteins had N-terminally orientated intact GST-protein fusion partner. In both procedures described, the carboxyl terminus of α1I domain contained ten (VDSSGRIVTD) and α2I domain six non-integrin amino acids (EFIVTD). In publications I and II the I domains were labeled directly with Europium before use in binding experiments. For these experiments see chapters 4.2.4., and 4.2.5.

4.2.2 Generation of mutated α2I domains (II)

Site-specific mutations in the α2I domain were made using the Stratagene QuickChange mutagenesis kit (Stratagene), essentially following manufacturer's instructions. For wild-type construct generation see chapter 4.2.1. The mutations produced following amino acid substitutions: Asp219->Ala, Asp219->Asn, Asp219->Arg, Glu256->Gln, Asp292->Asn, and Glu299 -> Gln. To generate double mutation Asp219->Asn/Asp292->Asn, two sets of PCRs were carried out. For αC helix deletion, the primers produced amino acid deletion in residues 284-288 (GYLNR). The PCR reactions were performed using Pfu-polymerase which makes at 68 °C exactly one copy of the whole vector containing the α2I domain sequence. The PCR-product was digested with DpnI enzyme, which cuts only methylated DNA, i.e. only the original PCR-template is digested. Production and purification of mutated α2I domains were carried out as described earlier in the context of wild-type α2I domain production in chapter 4.2.1. The α2I domains were separated from GST-fusion partner by thrombin cleavage and further labeled directly with Europium for binding experiments. For these see chapters 4.2.4, and 4.2.5. For IAsys biosensor

experiments they were used as GST-fusion proteins. For these see chapter 4.2.8. The recombinant products mutated by point substitutions were called according to amino acid replacement as follows: D219A, D219N, D219R, E256Q, D259A, D259N, D292N, E299Q and α2D219N/D292N. The αC helix deletion mutated recombinant protein was called ΔαCα2I.

4.2.3 Generation of human recombinant α10I domain as MBP-fusion protein (IV)

The α10I domain cDNA was generated by RT-PCR of mRNA isolated from KHOS-240 cells (Human Caucasian osteosarcoma, ECACC). The DNA fragments were purified and the insert was connected to the multiple cloning site of pMAL-c vector expression of α10I domain as a fusion with maltose binding protein (MBP). In sequencing, the substitution mutation (Thr -> Gly) in the nucleotide position 741 was discovered. This leads to amino acid change from phenylalanine to cysteine in amino acid position 222. The mutation was corrected by using QuickChange Site-directed Mutagenesis Kit (Kebo) based on the principal described in the context of α2I domain mutations. For this see chapter 4.2.2. The production of MBP–α10I domain fusion protein was carried out by instructions given by NewEngland BioLabs as the expression system manufacturer. The expression was promoted by a *lac*operon inducer, IPTG. Cells were harvested, resuspended, sonicated and then centrifuged. Supernatants were pooled and amylose resin was added to harvest fusion proteins. Resin was transferred onto the chromatography columns and washed to remove contaminating proteins. The maltose was added to elute immobilized fusion protein competitively. The recombinant α10I domain produced was 328 amino acids long corresponding to α10 integrin sequence 165-492 (YMDVVI...SELCP). In addition, the protein had N-terminally orientated intact MBP-protein fusion partner. The C-terminal region contained no non-integrin amino acids. The α10I domain preparation was used after dilutions as such for binding experiments. For these see chapter 4.2.6.

4.2.4 Direct Europium labeling of αI domains (I, II)

Europium labeling was based on the covalent thiourea bond formation between free amino groups on the protein and the isothiocyanate group of N1-p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N4-tetraacetic acid chelated with Europium. Labeling was carried out by strictly following Wallac´s instructions as the manufacturer. For this, $NaHCO_3$ was added to the purified α1I and α2I domain preparation. For αI domain generation see chapter 4.2.1. The Europium labeling reagent was added and incubated. The unbound label was removed by adding mixture to gel filtration on a Sephadex G50/Sepharose 6B column. To find labeled positive fractions samples were taken from elution, enhancement solution was added, and time-resolved fluorometry was used to measure the Europium signal. Preparations with positive signal were used in experiments as such after dilution. For this see chapter 4.2.6.

4.2.5 Binding assay for directly Europium-labeled αI domains (I, II)

Precoating of a 96-well microtiter plates was done by exposure of 0.1-15 mg/cm^2 (0.3-45 mg/ml) monomeric collagens according to manufacturer's instructions. Coating was done with the following collagen subtypes: rat type I (rI), human type II (hII), bovine type II (bII), chicken type II (cII), human type III (hIII), mouse type IV (mIV), human type V (hV), and human recombinant type XIII (hXIII). The commercially produced and non-pepsin purified collagens were used after dilutions as delivered from manufacturers. The recombinant human ectodomain of collagen type XIII was produced as described in chapter 4.1.1. The blank wells were coated with 7.5 % BSA. As well, the residual protein absorption sites on all wells were blocked with this preparation to reduce non-specific binding. The Europium-labeled α1I and α2I domains (for labeling see chapter 4.2.4) were added at a concentration of 1-30 mg/ml (40-1200 nM) containing $MgCl_2$ to the all wells and incubated. In one set of experiments in publication II with mutated α2I domain, the Mg^{2+} concentration was varied or EDTA to chelate all the Mg^{2+} was added to the suspensions. In all experiments, the wells were washed with PBS containing $MgCl_2$ to remove unbound αI domains. The enhancement solution was added to each well and incubated to release Europium from collagen: αI domain -complex. The Europium signal was measured by time-resolved fluorometry.

4.2.6 Binding assay for αI domains as GST or MBP fusion proteins (III, IV)

In publication III, precoating 96-well microtiter plates were done by exposure to either monomeric or fibrillar bovine collagen type I as described in instructions given by manufacturer. For collagen type I fibrillogenesis and sample preparation see chapter 4.1.3. In publication IV, the coating of microtiter plate was done by exposure to monomeric collagen types I (rI) or II (hII) as positive controls or either native or heat denatured rCOL15 domain of collagen type XVII. The commercially produced and purified collagens were used after dilutions as delivered from manufacturers. For rCOL15 generation and sample preparation see chapter 4.1.2. In both procedures, blank wells were coated with 7.5 % BSA to reduce non-specific binding. As well, the residual protein absorption sites on all wells were blocked with this preparation. In publication III, the recombinant proteins, α1I-GST and α2I-GST, were added to the coated wells at a concentration between 10 and 500 nM and removed after incubation. For α1I-GST and α2I-GST preparation see chapter 4.2.1. Europium-labeled GST antibody was added to the wells. In publication IV, the recombinant proteins, α1I-GST, α2I-GST or α10I-MBP were added to the coated wells at a concentration of 200 or 300 nM. For α10I-MBP preparation see chapter 4.2.3. The GST antibody or MBP antibody added to wells and incubated. Europium-labeled protein-G was then added. In both experimental procedures described concentrations of αI domains were chosen to achieve the saturation of binding, the stages involving αI domain were performed in the presence of $MgCl_2$ and all the wells were washed after each binding step. The solution was added to each well and incubated to release antibody or G-protein bound Europium. The Europium signal was measured by time-resolved fluorometry. The binding assay was done at least in three parallel wells.

4.2.7 Electron microscopy to study collagen type I fibril-integrin αI domain interaction (III)

After 60 min reaction samples with fibrillar or monomeric collagen type I were spotted onto formvar/carbon-coated copper electron microscopy grids. For collagen fibrillogenesis and sample preparation see chapter 4.1.3. Either α1I or α2I domain were allowed to attach to the collagen coated grids. For αI domain preparations see chapter 4.2.1. Control grids without αI domains were incubated in PBS. All steps stated were performed at 4 °C for monomeric and at 37°C for fibrillar collagen samples. All grids were washed and fixed. Then samples were washed and blocked in FBS. The GST antibody in presence of FBS was allowed to attach to the collagen bound αI domains. Grids were washed with PBS and incubated in PBS 1:55 diluted protein A-gold -conjugate solution. This conjugate preparation stated was a kind gift from Dr. George Posthuma (University Medical Center Utrecht). All sample grids were washed and embedded with methyl cellulose and stained with uranyl acetate. The samples were then examined by transmission electron microscopy. The αI domain linked gold particles attached to the collagen were counted from collagen fibrils as bound particles per μm of collagen fibril.

4.2.8 IAsys and BIAcore biosensor experiments (II)

IAsys-experiments were performed on the IAsys Auto plus apparatus. The activated IAsys carboxymethyldextran cuvette was coated with type I collagen as monomer with an amine-coupling kit according to manufacturer's instructions. In this procedure collagen amino groups are coupled to carboxylate groups of carboxymethyl dextran (CMD) at cuvette surface via EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide) chemistry. For coupling the non-pepsin purified rat collagen type I in Na-acetate solution was used. Collagen was precoated to the cuvette up to the saturating level based on the reading of the apparatus. For real time binding curve and kinetics, a concentration series (10–300 mg/ml) of α2I-GST wild-type and mutant α2I-GST fusion proteins were allowed to attach to the immobilized collagen in presence of $MgCl_2$. For generation of these recombinant αI domains see chapters 4.2.1. and 4.2.2. Results were analyzed with IAsys Fastfit software. In addition, the data concerning GST/α2I E256Q mutant was supplemented with BIAcore real time protein-protein interaction assay analogous to the described IAsys system. The non-pepsin purified rat collagen type I was immobilized on flow cell of a CM5 sensor chip as well as in using an amine-coupling kit. The GST tag was first removed and then α2I domain without fusion partner was injected as analyte at concentration of 100 μg/ml in the presence of $MgCl_2$.

4.2.9 Molecular modeling

A three-dimensional (3D) model of the structure of the ΔαCα2I domain was built using MALIGN (Johnson & Overington 1993, and Johnson et al. 1996) in the BODIL modeling package (unpublished results) and MODELLER 4.0 (Sali & Blundell, 1993). The model was constructed almost entirely from the x-ray structure of the α2I domain (Emsley, 1997), but the αMI (Lee et al. 1995) and αLI (Qu & Leahy, 1995) domains were used to build the structure in the vicinity of

the deleted helix. This was done because the number of the residues in that area in ΔαCα2I is exactly the same as in αM and αL. This makes the modeling more reliable in that region. The x-ray structures were obtained from Protein Data Bank (Bernstein et al. 1977).

4.3 Studies on cell-collagen interactions (I-IV)

4.3.1 Transfection of CHO cells to express α1β1 or α2β1 integrins (I-IV)

The collagen binding integrin negative wild-type chinese hamster ovarian cells (CHO) were obtained from the American Type Culture Collection (ATCC; CCL-61). They were used as hosts for stable transfection and expression of human integrin α1 or α2 subunits. The integrin β1 subunit is expressed endogenously by these cells. The α1 integrin cDNA (Briesewitz et al. 1993) was kindly provided by Dr. E. Marcantonio (Columbia University). The α2 integrin (Takada & Hemler 1989) cDNA was kindly provided by Dr. M. Hemler (Dana-Farber, Cancer Institute). The expression plasmid consisting of human α2 integrin subunit cDNA in the pAWneo2 vector (Ohashi et al. 1985), a kind gift from Dr. A Weiss (University of California), was used to transfect CHO cells. Electroporation was used for this with apparatus according to manufacturer's instructions. As well, the expression plasmid consisting of human α1 integrin subunit cDNA in the pLEN -vector (Dickeson et al. 1999) was co-transfected with pAWneo2 vector using electroporation. For vector control, the cells were transfected with pAWneo2 plasmid only. The cell surface expression of α1 or α2 integrin subunits were analyzed by flow cytometry. For CHO α1 cells commercial primary α1 integrin subunit antibody (anti-rat/mouse;) or antibody SR-84 (anti-mouse), a kind gift from Dr. W. Rettig (Boehringer Ingelheim), was added to the reaction. For CHO-α2 cells commercial primary α2 integrin antibody (anti-mouse) or antibody 12F1 (anti-mouse), a kind gift from Dr. V. Woods (UCSD) was added. For negative control reactions PBS was added instead of antibody. The integrin bound SR-84 and 12F1 primary antibodies were stained with rabbit anti-mouse IgG coupled to flourescein. For the commercially obtained primary antibodies FITC-labeled rabbit anti-mouse IgG was used. In order to measure the amount of expressed collagen binding integrins on the cell surfaces, the flourescein or FITC excitation spectra was analyzed by using a FACScan or FACSCalibur apparatus. For the measurement the CHO cell preparation expressing α1β1 integrin, called CHO-α1β1#5, was a mixture of cell clones and used as such. Three independent single cell clones expressing α2β1 integrin, preparations called CHO-α2β1#3, CHO-α2β1#9 and CHO-α2β1#12, were used. For assays with these cell lines see chapters 4.3.3. and 4.3.4.

4.3.2 Generation of CHO cell line to express mutated α2β1 integrin (II, III)

The wild-type construct containing α2 integrin cDNA in the pAWneo2 vector was generated as described in chapter 4.3.1. To produce a more easily remouldable unity, the insert was foreshortened and further cloned temporarily to the pGEX-4T-3 vector. This wild-type construct was digested with enzyme

having restriction sites in upstream from XbaI site in pAWneo2 vector multiple cloning site and in the α2 integrin cDNA insert, therefore generating a DNA stretch ("1 918 bp unit") containing the αI domain area to be mutated. In addition, the rest of insert and the most of vector containing fragment of 8442 bp ("8 442 bp unit") is formed. Linearized pGEX-4T-3 vector and αI domain area containing "1 918 bp unit" was ligated. Site-specific mutations in the αI domain area of "1 918 bp unit" insert were made using the Stratagene QuickChange mutagenesis kit , essentially following the manufacturer's instructions based on the principal described in chapter 4.2.2. For this, the Pfu enzyme based site directed mutation PCR were carried out to generate either Asp219-> Asn or Asp292 -> Asn point mutation. Two sets of PCR-reactions to generate Asp219 -> Asn/Asp292 -> Asn double mutation were done. The construct was digested with BamHI enzyme to release mutated "1 918 bp unit" insert. The fragment was ligated to original position to the "8 442 bp unit", which contains the pAWneo2 vector sequence and the rest of α2 integrin subunit cDNA. The transfection of CHO cells by electroporation and the measurement of double mutated α2 integrin subunit expression on the cell surface were carried out as described in chapter 4.3.1. Single cell clones called α2D292N and α2D219N were studied. Two independent cell clones expressing double mutated α2β1 integrin, preparations called α2D219N/D292N#2 and α2D219N/D292N#4, were used in parallel. For spreading and contraction assays utilizing these cell lines see chapters 4.3.3. and 4.3.4., respectively.

4.3.3 Cell spreading assays (I-IV)

In publications I and II, the precoating of 96-well microtiter plates was done by exposure to 0.1-7 μg/cm2 (0.3-21 μg/ml) monomeric collagens. The coating was done with the following collagen subtypes: rat type I (rI), bovine type I (bI), human type II (hII), bovine type II (bII; a kind gift from Dr. M. van der Rest), chicken type II (cII), human type III (hIII), mouse type IV (mIV), human type V (hV), and recombinant human type XIII (hXIII). The type hXIII was produced as described in chapter 4.1.1. In manuscript III, the universal 96-well plates were precoated with either monomeric or fibrillar bovine collagen type I. For collagen fibrillogenesis and sample preparation see chapter 4.1.3. In publication IV the plates were precoated overnight in PBS with commercial monomeric collagen type I from rat and type IV from mouse for positive controls and with either native or heat-denatured recombinant COL15 (rCOL15) of human collagen type XVII. For rCOL15 collagen preparation see chapter 4.1.2. The commercially produced and non-pepsin purified collagens were used after dilutions as delivered from manufacturers. In all experiments, the blank wells contained PBS during precoating and the residual protein absorption sites on all the wells were blocked with heat inactivated FBS. Cells were allowed to attach and spread in the wells for 40, 80, or 120 min either in serum free medium containing cyclohexamide to prevent endogenous matrix synthesis. In all publications, the α1 (clone CHO-α1β1#5) and/or α2 (clones CHO-α2β1#3, CHO-α2β1#9 and CHO-α2β1#12) integrin subunit transfected cells were used. For cell transfections see chapter 4.3.1. In publication II, also the mutated α2 integrin subunit expressing cells (clones α2D219N, α2D292N, α2D219N/D292N#2 or α2D219N/D292N#4) were used. For generation of mutations see chapter 4.3.2. In addition to cells transfected, in publication IV, the immortalized human

keratinocyte cell line (HaCaT) was studied. This cell line was obtained from Dr. N. E. Fusenic (DKFZ). In some spreading experiments, the contents of suspensions were varied with antibodies and synthetic peptides. In one set of experiments in publication I, the 1 μg/ml of function blocking α2 integrin subunit antibody 5E8, a kind gift from Dr. R. Bankert (Rosswell Park Cancer Institute), was present during the incubation. In publication IV, the role of specific integrin subunits in HaCat cell adhesion to rCOL15 was tested by adding to cell suspensions integrin function-blocking antibodies against either the β1, αV, α2, α3, or α5 integrin subunits. In inhibition studies, either linear or cyclic synthetic peptides were added to the cell suspensions. Linear peptides (P1-P22) corresponding to COL15 sequence were used. The cyclic RGD-peptide contained a covalent bond between the cysteins. A similar RGE-peptide was used as a nonfunctional control. In all studies, the medium containing non-adherent cells was poured out without extra washes. Adhered cells were fixed. The cells were examined and photographed under phase contrast microscope. The total number of cells attached and the ratio of spread cells were counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus.

4.3.4 Collagen gel contraction assay (III)

Vector transfected chinese hamster ovarian cells (CHO) or cells expressing either α1β1 integrin (clone CHO-α1β1#5), α2β1 integrin (clone CHO-α2β1#12) or double mutated α2β1 integrin (clones CHO-α2D219N/D292Nβ1#2 or CHO-α2D219N/D292Nβ1#4) were used. For integrin subunit transfections see chapter 4.3.1. and 4.3.2. Cell suspensions were mixed with medium and Hepes neutralized collagen type I solution. The mixture was transferred into each well in a 24-well cell culture cluster and allowed to clot. The edges of the gels were detached by needles from the sides of the wells and αMEM medium was added. After three days, the wells were photographed, and the reduction in surface area was measured.

4.3.5 Lateral cell migration assay (IV)

A 24-well cell culture cluster was precoated with human fibronectin or either native or heat denaturated rCOL15; all matrixes in concentration of 5 μg/cm^2. For rCOL15 collagen preparation see chapter 4.1.2. Semiconfluent HaCaT cells, obtained from Dr. N. E. Fusenic (DKFZ), were counted and suspended in serum-free medium, and transferred into a custom-made stainless steel cylinder with an opening of 2.8 mm in diameter towards the precoated matrix on the bottom of the wells. The cells were allowed to attach to the substrates. Cylinders were removed, nonadhered cells were washed away, and the adhered cells were then allowed to migrate for 2 or 4 days. Cells were fixed, stained with crystal violet, and washed. The rate of migration was estimated by measuring the surface area (mm^2) covered by the cells.

5 RESULTS

The results presented in following are reviewed as conclusions in Fig. 5 in the end of this section.

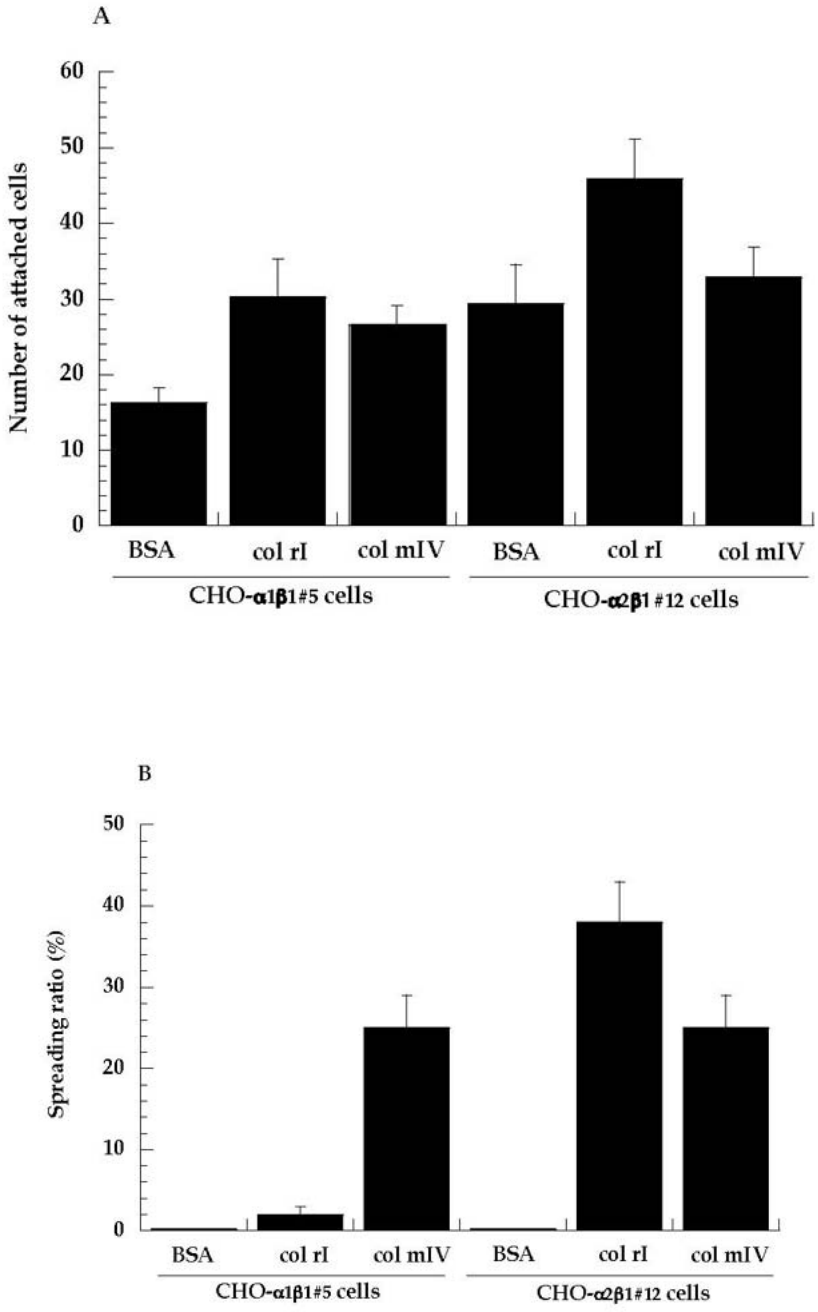
5.1 Collagen binding profile of α1β1 integrin is different than α2β1 integrin (I)

5.1.1 Methods point of view

All the binding and spreading experiments presented is this thesis are based on observations presented below. According to them the cellular spreading behavior is time-dependent on collagenous ligands and mediated specifically by collagen binding integrins. Furthermore, the integrin recognition of collagens is mediated by integrin αI domains even though the saturation in binding was not achieved and therefore kinetic parameters could not be detected. For results see Fig. 1/I, 2/I, 3/I, and supplementary Fig. 1 and 2. In addition, Table II/II and Fig. 3A/II and 2B/III are referred in this context.

For cellular studies, the endogenously integrin β1 subunit expressing chinese hamster ovarian cells (CHO cells) were transfected to express in addition either intact human integrin α1 or α2 subunits. The vector transfected CHO cells did not express any collagen binding α subunits, the control cell spreading on collagen type I being comparable to cell spreading on bovine serum albumin (BSA) as negative control (Fig. 1B/I). As well, the BSA does not induce the notable spreading of integrin α2 subunit transfected cells (Fig. 1D/I). The expression levels of integrins on the surfaces of the transfected cells were tested by flow cytometry. The α1β1-cell population, called CHO-α1β1#5, was found out to be mixture of two or three cell clones (Fig. 2A/I), therefore having the cells with different integrin α1 subunit expression levels. Two single α2β1 cell clones examined, called CHO-α2β1#3 and CHO-α2β1#12, were found to express integrins about equally (Fig. 1A/I). For CHO-α2β1#12 cell expression see Table II in publication II. As preliminary experiment, the time dependence of CHO-α2β1#3 of cell attachment and spreading on collagen was measured by allowing them to spread for different times on rat collagen type I. The spread

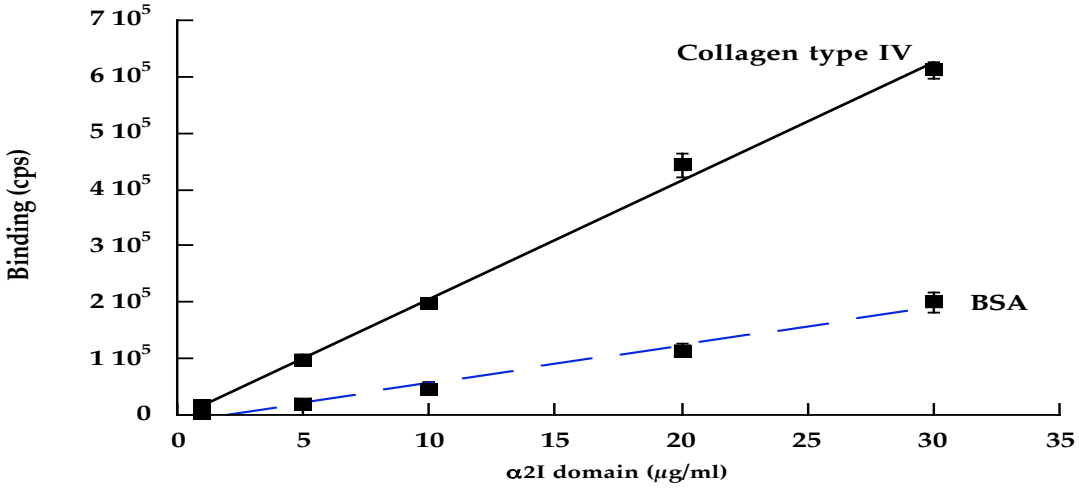
cells were defined to have clearly visible cytoplasm around the nucleus being typically stretched, spindle-like cells, and shimmering in blue color under phase-contrast microscope. For similar morphology, see spread CHO-α1β1# 5 cells on collagen type IV in Fig. 3A/I. In these preliminary studies, only fibroblast-like spreading morphology was detected. The attached but non-spread cells were round or splinter-like, shimmering in yellow or only faintly in blue (not shown). The used collagen concentration was observed to be suitable for spreading induction and the clear time-dependance on this movement was discovered (Fig. 1C/I). Due to most definable changes in cell morphology after 120 min incubation time and in order to detect slowly structural changes, it was chosen as observation point for further studies. In the presence of function blocking α2 integrin subunit antibody this spreading of α2β1 expressing cells was completely blocked (Fig. 1B/I), indicating the existence of collagen binding integrin mediated spreading behavior. The measurement of spreading as described above were chosen instead of adhesion studies used in several reports. In later, the data obtained from experiments is based on absorption of crystal violet stain of all attached cells, which is further measured spectrometrically after cell lysis. For example see Ivaska et al. (1990a). However, in our preliminary experiments, the total number of attached cells during incubation did not correlate satisfactorily, unlike the spreading behavior, with the results previously reported of collagenous specificity (supplementary Fig. 1A and 1B).



SUPPL. FIGURE 1 **Total number of attached cell does not correlate with active cell recognition and spreading behavior.** In cell spreading assay the microtiter plate was precoated with purified rat collagen type I and mouse collagen type IV. Residual binding sites were blocked with BSA. BSA was used as background control. Cells were allowed to attach and spread followed by fixing. In panel A the total number of attached cells were counted. In panel B the total number of attached and spread cells were counted and perceptual ratio of spreading were calculated. In both measurements the data is mean ± S.E. of three parallel measurements.

For solid phase binding studies the I domains of human integrin α1- and α2-subunits, generally accepted to be essential for collagen type I and IV primary recognition and binding (for example see Calderwood et al. 1997), were produced as recombinant GST fusion proteins. In native polyacrylamide gel electrophoresis one extra band with a heavier molecular weight was seen in the α2I domain lane and it was necessary to treat the sample with dithiothreitol to get one band alone with properly folded I domain. This phenomenon was also observed in publication II , but reported first by Ivaska et al. (1999a). The recombinant αI domains were labeled with Europium. In solid phase binding assay they were allowed to attach to immobilized collagens and the bound signal was measured afterwards as time-resolved florescence counts per second (cps). In all the experiments, the αI domain bound to rat collagen type I as positive control and the background binding to BSA were measured. Due to the fact that the collagen binding activity of integrin I domains has been shown to be Mg^{2+} -dependent, the assays were carried out in the presence of $MgCl_2$. In preliminary binding experiments with α1I and α2I domains, the amount of collagen types I and IV attaching to the well surface was discovered to saturate at precoating concentration of 3-5 $\mu g/cm^2$. For this type of experiment see figure 3A for example in publication II. Based on this finding, the matrix precoating concentration was chosen to be 5 $\mu g/cm^2$ for further binding experiments. In the second set of method testing, the saturation of binding was not achieved despite the α2I domain concentration was highly increased on rat collagen type I coated surface (supplementary Fig. 2).

The relation between αI domain concentration and collagen type IV bound αI domain was linear. However, the αI domain dilution produced clearly measurable time-resolved signal and the non-specific binding to BSA was less than 10 % of maximal collagen binding (not shown). Based on this, the αI domain concentration of 40 nM was chosen as an observation point for further experiments. According to solid phase binding measurements based on GST/α2I domain in publication III, this concentration represents nearly the saturating level (Fig. 2B/III). Some difference in the number of Europium label coupled to αI domains, indicated by the difference in total cps given by the same protein amount in fluorometrical measurement, were detected in different protein stocks labeled separately (not shown). This variation had, however, not affected the relative αI domain binding of collagen (not shown).



SUPPL. FIGURE 2 **No saturation in binding is achieved with direct Europium labeled αI domain.** In solid phase binding assay the microtiter plate wells were precoated with mouse type IV collagen. Residual binding sites were blocked with BSA. As well, the BSA was used as a background control. Europium-labeled human recombinant α2I domains were allowed to attach in the presence of $MgCl_2$. Wells were washed and the bound Europium signal was measured with time-resolved fluorescence. The data is mean ± S.E. of three parallel measurements.

5.1.2 The fibrillar collagens are recognized differently by integrins regardless of known integrin binding sequences

Two integrin-type collagen receptors, α1β1 and α2β1, are structurally very similar. However, cells can concomitantly express both receptors and they might have independent functions as indicated by several reports dealing with collagen type I and IV binding. However, the integrin-type receptors for most collagen types are unknown. Due to this, the cell spreading studies were carried out on fibrillar collagen type II, III, and V monomers in addition to control collagen types I and IV. These experiments revealed that the existence or absence of reported integrin recognition and binding motifs in collagens does not necessary predict the integrin function and collagen binding integrins do have different ligand specificity. For results see Table I/I, and Fig. 1D/I, 2B/I, and 3/I. Results are summarized in Table 2.

The rate of spreading with CHO-α1β1#5, CHO-α2β1#3 and CHO-α2β1#12 cells were detected on rat collagen type I and mouse collagen type IV, and experiments were typically repeated due to variation in parallel experiments (Table I/I). As suggested earlier, the α1β1-integrins favor type IV collagen over fibrillar collagens. During 120 min incubation more CHO-α1β1#5 cells had spread on type IV than on type I collagen in four out of five measurements (Table I/I). Correspondingly, more CHO-α2β1 cells from clones tested had spread on collagen type I than on collagen type IV in four out of five studies. The slower CHO-α1β1 cell spreading detected was perhaps due to the existence of lower integrin expression level. Furthermore, the "mute" insert containing

cell selection and the gradual decrease in total expression of α1 subunit in the cell population was detected during long-term cultivation (not shown). The rat collagen type I α1(I)-chains (GCRT1S) have reported to include one GLPGER- and one GFPGER-motif, and α2(I)-chain (AF121217) with two GLPGER-motifs. These repeats can act as α1β1, α2β1, and α11β1 integrin recognition and binding sequences after potential proline hydroxylation as part of post-translational modification (Knight et al. 1998, Knight et al. 2000, and Xu et al. 2000, Zhang et al. 2003). Mouse collagen type IV includes one GFPGER-motif in α1(IV)-chain (P02463). This motif is found in many, but not all, of the vertebrate collagen sequences. In addition to collagen types I and IV, CHO-α2β1#12 cells can spread effectively on bovine collagen type II, human collagen type III, and human collagen type V. After 120 min incubation, the spreading detected on substrates stated were 60±5, 72±7, and 48±9 %, respectively (Fig. 1D/I). As well, the bovine collagen type II α1(II)-chain (CGBO6C) contains potentially active integrin recognition GLPGER motif. Interestingly, human collagen type III α1(III)-chain (AAL13167) and any of human type V α(V)-chains (P20908, P05997, XM009025), do not contain any known integrin binding sites. The CHO-α1β1#5 cell spreading ratio on collagen human type III was 29±5 % (Fig. 2B/I), further indicating slower spreading. No α1β1 cell spreading on bovine cartilage collagen type II and human type V was detected with this amount of substrate available (Fig. 2B/I). The collagen type II experiment was repeated with human origin collagen, but still only minor effect comparable to spreading on BSA was detected (not shown). It is notable that, as reviewed in chapter 2.1.1.2, the human collagen type II α1(II)-chain (P02458) contains both GLPGER and GFPGER binding motifs, which however seem to be inactive in this context. Based on these negative findings, a second set of experiments were carried out. The precoating was done with five-fold higher concentration to saturate well surface. The collagen types rI, hII, mIV, and, hV were used and these immobilized ligands were introduced again for CHO-α1β1#5 cells (Fig. 3C/I). In general, the amount of cell spreading were found to be directly proportional to substrate concentration. When substrate concentration of control ligands were increased five-fold, the maximal CHO-α1β1#5 cell spreading on collagen rI and mIV was enhanced to 37±5 and 80±10 %, respectively. Interestingly, also on collagen type hV was detected spreading of 34±5 %. Furthermore to increase in spreading ratio, changes in cell motility was discovered and two morphologically distinct groups could be observed. In addition to fibroblast-like cells described (Fig. 3A/I) before, the circular or slightly polygonal, flattened cells with a string of pearl-like plasma membrane structures occurred. For similar morphology see spread CHO-α1β1#5 cells on collagen type XIII in Fig. 3B/I. If spread cells were divided into fibroblast-like cells and circular flat cells the numbers were: fibroblast-like:13±2 % (type I), 60±4 % (type IV), 19±4 % (type V); and circular flat cells: 24±5 %, 20±1 % and 15±5 %, respectively (Fig. 3C/I). In other words, the α1β1 subunit can mediate spreading also on collagen type V, but ligand concentration needed for induction was higher than other collagen subtypes tested or what CHO-α2β1 cells needed. Importantly, despite the higher ligand concentration, there was no still spreading of CHO-α1β1#5 cells on human collagen type II .

5.1.3 Collagen recognition of α1I domain alone is not always sufficient to enhance integrin mediated cell spreading

To study which collagen types are primarily recognized by α1 and α2 integrin subunits, the recombinant αI domain based binding measurements were carried out. Particularly, the collagen type II was studied. In these studies, the αI domain binding did not correlate exactly with spreading behavior and the recognition of collagen by αI domain alone is not always enough to mediate regulatory effects leading to cell spreading. For results see Table I/I, and Fig. 1D/I, 4B/I, and 5B/I. Results are summarized in Table 2.

The relative binding of recombinant α1I domain for precoated collagen types rI, hIII, mIV, and hV were 100±12, 91±6, 116±4, and 87±9 arbitrary units (Fig. 4A/I), respectively. According to this assay, the difference between the fibrillar collagen type I and the network forming type IV collagen were relative small, if compared to the ratio indicated by spreading assay with CHO-α1β1#5 cells (Table I/I). The relative α2I domain binding to collagen types stated were 100±4, 108±3, 126±11, and 46±3 arbitrary units (Fig. 4B/ I), respectively. As well in the case α1I domain and CHO-α1β1 cells, the α2I domain binding data cannot explain all the differences detected in the spreading behavior of CHO-α2β1#3 and CHO-α2β1#12 cells (Table I/I). In the case of α2I domain binding assay, it is especially notable, that the binding to collagen type mIV was preferred over collagen type rI binding. To study further the fact that CHO-α1β1#5 cells, unlike CHO-α2β1 cell clones, could not spread on type II collagen, additional set of binding of studies were carried out on three different origin type II preparations (Fig. 5A/I and 5B/I). A large amount of α1I domain (Fig. 5A/I) was found to bind to human collagen type II, when compared with collagen type I binding (Fig. 5A/I). This finding was verified in an independent assay (not shown). The α1I domain binding to chicken and bovine type II collagens was still measurable but clearly smaller. This result indicates, that human collagen type II is recognized by human integrin α1I domain with the same efficiency than the other fibrillar collagens and therefore αI domain function cannot explain the lack of CHO-α1β1#5 cell spreading on human origin collagen despite the increased in ligand concentration. The α2I domain was less selective in collagen recognition. It bound relatively large and about equal amounts to human, chicken, and bovine type II collagens tested (Fig. 5B/I). This relative binding ratio on bovine collagen type II is concordance with data obtained from CHO-α2β1 cell spreading studies (Fig. 1D/I).

5.1.4 The α1β1 integrin can recognize transmembrane collagen type XIII

The transmembrane type XIII collagen recognition by the integrins has not been tested before. For spreading and binding assays, the ectodomain of human collagen type XIII was expressed in insect cells with a baculovirus based system. According to this, the α1β1 integrin, unlike α2β1 integrin, was able to recognize and mediate cell spreading on this recombinant collagen with distinct cell morphology. For results see Fig. 3/I, and 4/I. Results are summarized in Table 2.

In this study, the CHO-α1β1 cells could attach to and spread effectively on collagen type hXIII. The CHO-α1β1#5 cell spreading on this recombinant protein was during a 120 min incubation only a little bit slower than spreading

on mouse collagen type IV (Fig. 3C/I). However, the morphology of most spread cells on collagen type XIII was different when compared with the cell spreading on type mIV collagen. Interestingly, most of cells were circular (64±4 %; Fig. 3B/I and 3C/I) and only a few fibroblast-like cells were seen (5±5 %) as in the phenomenon described in chapter 5.1.1. The number of CHO-α2β1#12 cell spreading on collagen type hXIII (3±2 %; Fig. 3D/I) was comparable to cell spreading on BSA as a negative control (not shown), suggesting that α2β1 integrin cannot effectively mediate cell adhesion to collagen type hXIII. If the collagen precoating concentration was decreased by a fifth, no spreading of either of cell lines were detected on collagen hXIII (not shown). The results from αI domain binding studies are in accordance with these spreading observations described. The amount of α1I domain bound to recombinant ectodomain of type hXIII collagen was found to be even higher (Fig. 4A/I) than to collagen types rI and mIV. This confirms the suggestion that α1β1 integrin is collagen type hXIII collagen-binding protein. Importantly, only marginal binding of α2I domain, comparable with binding to BSA, was detected to collagen type hXIII (Fig. 4B/I), suggesting that α2β1integrin dimer cannot recognize this collagen type. Collagen typehXIII α1 chain peptides (NM005203) does not contain any known integrin binding motifs.

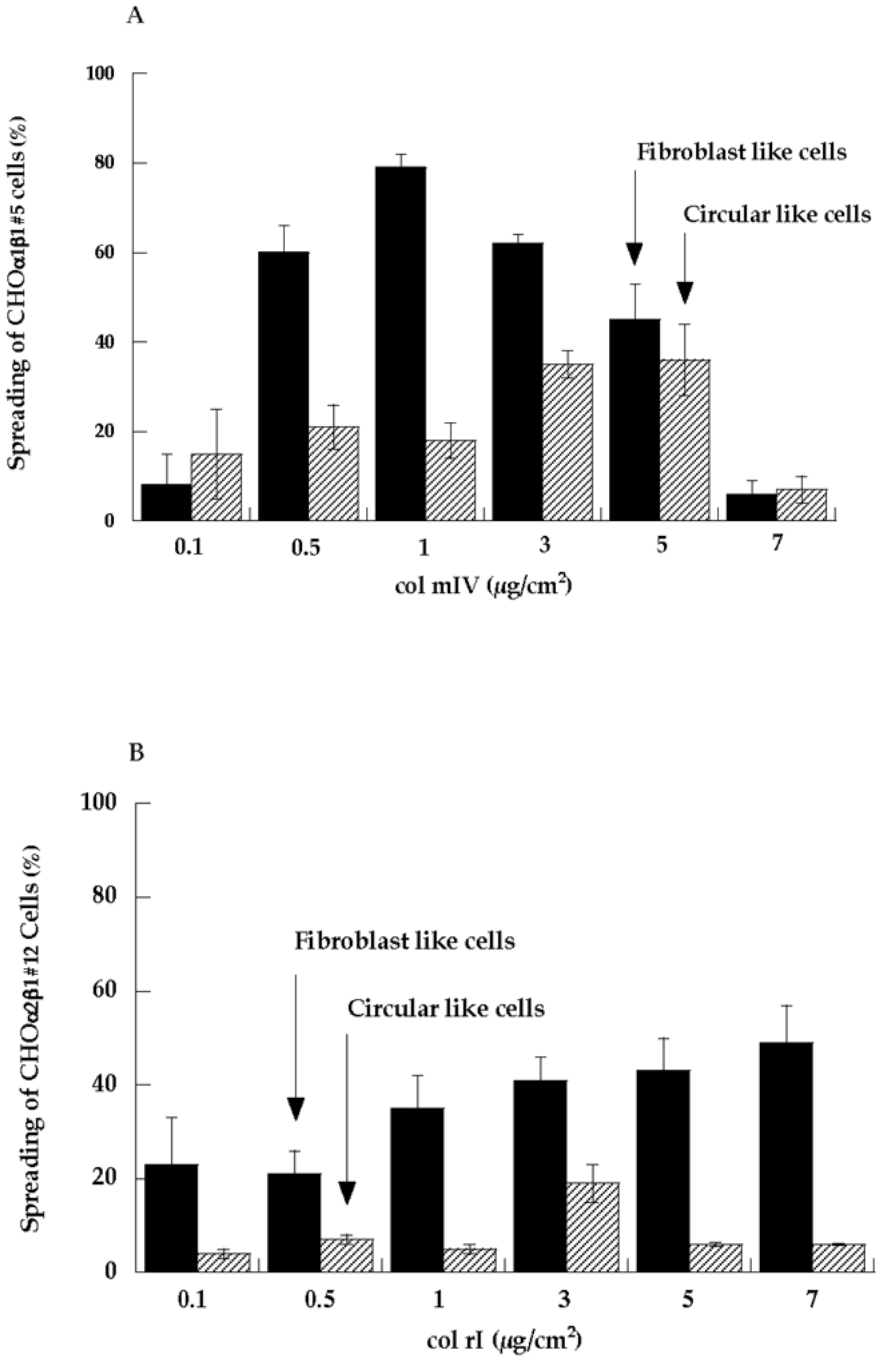
TABLE 2 The αI domain binding and cell spreading data from original publication I reviewed. The relative αI domain binding to collagen type I is presented in percential *em quadrats*. From spreading data the maximal spreading detected is shown. Known integrin binding sites after potential proline hydroxylation (P*) in collagen α chains are stated. Collagen origins: r = rat, c = chicken, b = bovine, m = mouse, and h = human.

Collagen type ($\mu g/cm^2$)	α1I domain binding relative to collagen type I binding (%)	α2I domain binding relative to collagen type I binding (%)	CHOα1β1-cell spreading (max. %)	CHOα2β1-cell spreading (max. %)	Known integrin recognition sites in collagen α-chains
rI (1) rI (5)	Not measured 100	Not measured 100	18±5 37±5	97±3 83±2	GLP*GER (α1(I)) GFP*GER (α1(I)) GLP*GER (α2(I)) GLP*GER (α2(I))
cII (5)	25-50	50-75	Not measured	Not measured	No sequence available
bII (1) bII (5)	Not measured 25-50	Not measured 50-75	0 Not measured	60±5 Not measured	GLP*GER (α1(II))
hII (1) hII (5)	Not measured ≥ 100	Not measured 75-100	0 0	Not measured Not measured	GLP*GER (α1(II)) GFP*GER (α1(II))
hIII (1) hIII (5)	Not measured 75-100	Not measured ≥ 100	29±5 Not measured	72±7 Not measured	No known motifs
mIV (1) mIV (5)	Not measured ≥ 100	Not measured ≥ 100	85±5 80±10	90±6 47±5	GFP*GER (α1(IV))
hV (1) hV (5)	Not measured 75-100	Not measured 25-50	0 34±5	48±9 Not measured	No known motifs
hXIIII (1) hXIIII (5)	Not measured ≥ 100	Not measured ≤ 25	0 69±5	0 0	No known motifs

5.1.5 The cell morphology is determined by collagen concentration and collagen subtype bound to the integrin

In order to study the ligand concentration dependence of morphological transition from fibroblast like to circular like cells, the collagen types rI and mIV were coated with different concentrations and spreading assays were carried out. The relation of cell morphology as function of substrate concentration is either linear or bell-shaped depending on collagen type surface. For results see supplementary Fig. 3.

On collagen type rI, both CHO-α1β1#5 cell and CHO-α2β1#12 cell spreading proportion after incubation was directly proportional to ligand concentration when fibroblast-like morphology was studied. The maximal spreading proportion were 7±3 % and 45±6 %, respectively. The circular-like spreading remained low, typically ≤ 5 %, and did not show precise concentration dependence with neither α1β1#5 or α2β1#12 cell line. On collagen type mIV, the relationship between the ligand concentration and spreading morphology of both cell lines were bell-shaped. This Gaussian type curve fitted to occurrence of fibroblast and circular-like spread cells similarly. Both maximal fibroblast (45±8 %) and circular-like (42±9 %) spreading was achieved with α2β1#12 cells at the concentration of 1 $\mu g/cm^2$. With minimal and maximal collagen type mIV concentrations the total spreading was decreased to about 10 %. Additionally the maximum existence of fibroblast-like α1β1#5 cells (70±5 %) was observed at the concentration of 1 $\mu g/cm2$. Most of the circular-like α1β1#5cells (35±9 %) were determined when ligand concentration was increased five-fold. The occurrence of both fibroblast and circular-like cells was directly proportional to incubation time (not shown); the fact tested with CHO-α1β1#5 and CHO-α2β1#12 cells on collagen type mIV. When spreading proportion detected here are compared to results presented in chapter 5.1.1., some variations are seen in the existence of different cell morphologies. However, in both sets of experiments, the proportion of total spreading and the relations between collagen subtypes are in accordance. This indicates the existence of stabile collagen recognition and binding mechanism, but different degree in outside-in signatory responses mediated by α1β1 and α2β1 integrin dimers.



SUPPL. FIGURE 3 **Morphology of spreading cells is dependent on collagen type and collagen concentration.** In cell spreading assay the microtiter plates were precoated either with purified rat type I (panel A) or mouse type IV (panel B) collagen as monomers. The residual binding sites were blocked with BSA. Cells were allowed to attach and spread for 120 min and fixed. Total number of attached cells and circular and fibroblast- like spread cells were counted and perceptual spreading proportion was calculated. The data in mean ± S.E. of three parallel measurements.

5.2 Integrin α2β1 recognizes collagen types I and IV by different mechanisms (II)

5.2.1 The α2β1 integrin specificity is partly determined by αC helix

The αC helix in the supposed ligand binding area is an unique structure shared by the collagen-binding α1, α2, α10, and α11 integrin subunit I domains. These independently folding inserted domains are proposed to determine at least partly the ligand specificity of integrin dimer. However, the αC helix participation in collagen binding has not been shown. To study function of αC helix in α2I domain, a helix deletion mutant was generated and the effects of this modification were studied to fibrillar collagen type I and network forming collagen type IV binding. Both IAsys and Europium labeled α2I domain based binding measurements were carried out. In order to estimate the effects of αC helix deletion the molecular modeling was used before experimental approach. Studies revealed an important role of αC helix in collagen type I binding and approved the different recognition mechanisms on collagen type I and IV binding. For results see Fig. 1A/II, 2/II, 3/II and 4/II.

Molecular modeling predicted that after removal of central set of five amino acids from αC helix, the surface of α2I domain would become more negatively charged and more flattened than the wild-type. In the x-ray structure of α2I domain, there is an Arg288 at the end of helix αC. This arginine residue forms a salt bridge with Glu318, situated between βF and helix α7. As a result of this deletion the Glu318 and the adjacent Asp317 are estimated to be placed in the vicinity of the MIDAS. Importantly based on experimental data, the αC helix deletion does not interfere in metal binding site (Fig. 4A-D/II) as presented in more detailed in chapter 5.2.2.

For experiments, the deletion between amino acids 284 and 288 (GYLNR) in α2I domain was generated. The recombinant αI domains were purified with a GST fusion partner to achieve greater mass and further easier detection by IAsys biosensor. The wild-type and the mutant recombinant were called GST/α2I and GST/ΔαCα2I, respectively. The concentration series of GST/α2I and GST/ΔαCα2I domains (10-300 μg/ml) were added into the reaction chamber and the binding to coated type I collagen was monitored in real time. For kinetics, see example in Fig. 2A/II. As expected, the GST/ΔαCα2I domain binding kinetics was different when compared to GST/α2I binding. The association and dissociation phase of the mutant was clearly faster than that of the wild-type, and the equilibrium of binding was reached more quickly (Fig. 2A/I). The total effect of these changes was seen on K_d values, which were estimated from binding at equilibrium (Req) *versus* ligand concentration. For GST/α2I and GST/ΔαCα2I domain the apparent K_d values were 90±30 nM and 430±90 nM, respectively (Fig. 2B/II). According to this αC helix deleted α2I domain can bind collagen type I about twice as much as wild-type αI domain at high concentrations (≥ 1500 nM). However, this variant collagen binding affinity is about five-fold weaker than wild-type binding. For the first time, this result demonstrates an important role of the αC helix in collagen type I binding. Furthermore, it confirms the previous suggestion that the collagen binding surface of α2I domain is located near the MIDAS site.

For further solid phase binding studies, the GST fusion partner was removed resulting in the proteins called α2I and ΔαCα2I, respectively. For detection they were labeled with Europium and the binding to rat collagen type I and mouse collagen type IV as monomers were tested in solid phase assay. The binding achieved was mainly directly proportional to collagen concentration used in the assays (Fig. 3A-C/II). The recognition of collagen subtypes by ΔαCα2I domain was different, if compared to wild-type α2I domain. The mutant preferred collagen type IV over collagen type I (Fig. 3B/II). Correspondingly, the wild-type α2I binding ratio to collagen type I was higher than to collagen type IV (Fig. 3A/II). This wild-type α2 subunit binding profile was presented on a cellular level in publication I. For this see chapter 5.1.1. These results indicate the different mechanism of type IV binding when compared with collagen type I binding. Interestingly, the collagen binding pattern of αC deletion mutant resembled the one previously described for α1I domain (Calderwood et al. 1997). As well, the similar binding profile was shown for wild-type α 1 subunit in publication I. This suggests, that the differences in αC helix structure might partially explain the functional differences between α1I and α2I domains by determining the part of their ligand specificity.

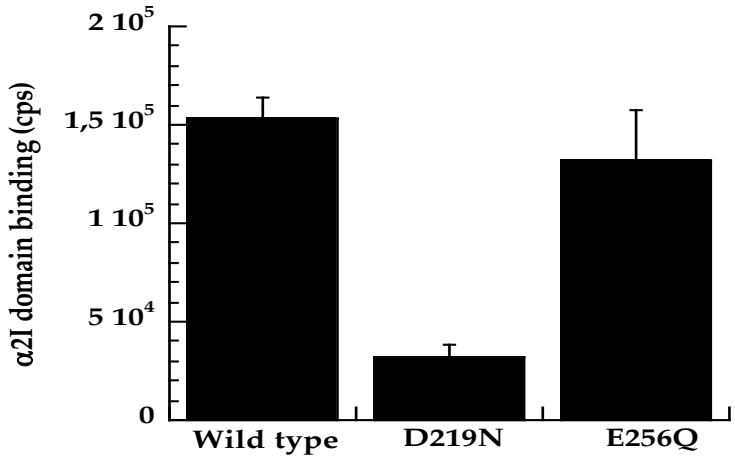
In collagen binding integrin αI domains, there is one extra amino acid residue in the loop between helices α3 and α4. In α1I and α2I domains, the extra amino acid has an opposite charge, namely Asp219 in α1I domain and Arg218 in α2I domain. This indicates another possible mechanism for the different ligand specificity of the two integrins. To test this possibility, the α2I domain having substitution from aspartic acid to arginine in position 219 was tested. This mutation did not, however, have a clear effect on the ratio of collagen type I /collagen type IV binding (Fig. 3C/II) in solid phase binding experiments. For α2I D219R in collagen I binding see chapter 5.2.2 indicated by biosensor experiments.

5.2.2 Negatively charged amino acids surrounding the MIDAS site are co-operatively involved in collagen binding

In addition to then crucial role of αC helix, it has been suggested that the charged amino acid residues surrounding the MIDAS site may play an important role in the recognition of collagen type I by integrin α2I domain. For example see publication by Pentikäinen et al. (1999). These negatively charged active residues are not, however, concerned to be the ones interacting directly with Mg^{2+} ion. The exact experimental evidence has been missing so far. To test this idea, five negatively charged amino acids were mutated in the area stated, and the effects on collagen type I binding were tested. This set of experimets was carried by using IAsys biosensor based binding assay. The data was supplemented by solid phase binding and cell spreading experiments. Four of the tested mutations in the vicinity of MIDAS affected significantly on collagen type I binding by α2I domain despite the fact that these modifications do not interfere with Mg^{2+} ion directly. On collagen type IV none of mutations tested had an effect on cell spreading, as well indicating the existence of different binding mechanism for collagen types I and IV. For results see Fig. 4/II, 6/II, Table I/II and II/II and supplementary Fig. 4.

For molecular tools, the nucleotide mutations were designed to produce

the following amino acid substitutions in α2I proteins to be secreted: D219A, D219N, D219R, E256Q, D259A, D259N, D292N and E299Q. The recombinant αI domains were expressed as a GST fusion proteins and purified as intact with the fusion partner (GST/α2I domain) for IAsys biosensor experiments. For solid phase binding experiments the GST-protein was further removed and remaining α2I domains were labeled with Europium for detection. In native gel electrophoresis every α2I domain protein sample had a small extra band having larger mass than the true α I domain band (not shown). These bands disappeared when conditions were reduced with dithiothreitol supplementation. This was observed in publication I and reported earlier by Ivaska et al. (1999a). The extra band is likely to be a small portion of misfolded protein that was able to refold back to correct conformation under reducing conditions. Due to the fact that the mutations were made close to MIDAS, it was necessary to check that none of these mutations affected significantly to the metal dependence of α2I domain binding. In addition to point mutations, the αC helix deletion presented in chapter 5.2.1., were tested in this set of experiments. The Europium labeled α2I domains were allowed to attach to immobilized type I collagen in the presence of four different Mg^{2+} concentrations. In addition, to reach the state of "0 mM" Mg^{2+}, EDTA was added into one αI domain dilution for each experimental set. The concentration of 2 mM Mg^{2+} represent satisfactory saturating conditions both with wild-type and all α2I domain mutants tested (Fig. 4A-D/II). The relative binding of mutants to collagen type I varied between 0.7 and 1.0 in the presence of this Mg^{2+}concentration if compared to wild-type α2I (ratio 1.0). The data indicates that neither point mutations stated, nor αC helix deletion, had effects on metal binding site. The GST/α2I domain wild-type and GST/α2I domain variant's binding to rat collagen type I monomer was monitored in real time by IAsys biosensor technology. For kinetic examples see Fig. 5/II. The results in the represented saturating conditions (300 μg/ml) are listed in Table I/II. The GST/α2I domain mutants D219A, D219N, D219R, D259N, D292N, and E299Q bound collagen type rI at 5-60 % of the wild-type GST/α2I domain levels (Table I/II). The aspartic acid substitutions in positions 219, 259, and 292 showed 65-95 %, 45-90 %, and 40-65 % decrease in binding, respectively. Further, glutamic acid substitution in position 299 produced a 70-90 % decrease. The results indicate that at least four negatively charged amino acids in integrin α2I domain, namely Asp219, Asp259, Asp292, and Glu299, have a significant role in collagen type I binding. It seems, however, that any of these residues does not alone determine the collagen recognition and binding mechanism. All the variants stated above gave satisfactory reproducible results in parallel experiments. Different to others, the binding of GST/α2I E256Q mutant to collagen type I varied between 35 and 100 % if compared to wild-type αI domain binding (Table I/II). The role of Glu120 in collagen binding was therefore studied further by removing first the GST fusion partner and then analyzing preparations binding ability by BIAcore technology and solid phase binding assays (not shown). In parallel, as a positive control in measurements D219N mutant was used α2I. In these experiments D219N variant showed a constant lowering of binding to collagen type I, the finding being in accordance with results from IAsys measurements (not shown). Further as an exception to other variants tested, the mutation α2I E256Q had no affect on collagen type I binding in these additional experiments (supplementary Fig. 4.).



SUPPL. FIGURE 4 **The E256Q mutation has no effect on collagen type IV binding.** In solid phase binding assay the microtiter plate wells were precoated with mouse type IV collagen overnight. The residual binding sites were blocked with BSA. Europium labeled wild-type α2I and D219N and E256Q mutated α2I domains were added in the presence of $MgCl_2$ into the wells. Wells were washed three times, and bound europium signal was measured with time resolved fluorescence. The data is mean ± S.E. of three parallel measurements.

Among other charged residues in MIDAS area, the amino acids Asp219 and Asp292 were found to be essential for α2I domain binding to collagen. Finally, the cell spreading experiments were carried out to test importance of these residues for the function in the complete integrin receptor. The D219N, D292N single mutations, or D219N/D292N double mutations were introduced in site-directed mutation PCR into full-length wild-type human integrin α2 subunit cDNA and transfected to CHO cells. The expression levels of mutated α2β1 integrins were analyzed by flow cytometry (not shown). Two clones of double mutated CHO cells, called α2D219N/D292N#2 and α2D219N/D292N#4, were used. One subclone of both α2D219N and α2D292N cells were analyzed. The expression ratios are shown as mean fluorescence value and available in Table II/II. In addition, the D219N cell population contained some α2 integrin subunit negative cells. Therefore the cells were stained with α2integrin antibodies, and the proportion of spreading was examined from positive cells under fluorescence microscope. To test the function of mutated integrins, the spreading of cells plated on rat collagen type I and on mouse collagen type IV was measured in spreading assay. In four parallel experiments the wild-type spreading proportion on collagen type I was between 0.67±0.05 and 0.84±0.04. The ligand concentration dependence in spreading behavior was not observed with concentrations used and the spreading morphology detected in this set of experiments was solely fibroblast-like. Compare to results in chapter 5.1.1. Neither of the cell clones harboring a single point mutation showed any significant alteration in spreading rate (Table II/II). The both CHO cell clones

expressing the doubly mutated integrin α2 subunit spread on collagen type rI remarkably more slowly than the wild-type CHO-α2β1#12 cells (Table II/II, Fig. 6A/II). The α2D219N/2D292N#2 and α2D219N/2D292N#4 cell spreading was comparable to spreading observed with wild-type CHO-α2β1#9 cells with 10-fold less integrin α2 subunit expression (not shown). The fact that one mutation is not alone enough to cause significant changes in this integrin function supports the idea that several charged amino acids in the α2I domain participate co-operatively in collagen type I binding. Interestingly, no differences in cell spreading were seen on collagen type IV (Fig. 6/II, Table II/II). The wild-type spreading detected on collagen type IV was between 0.54±0.09 and 0.91±0.03. Correspondingly, the spreading proportion observed with mutated integrins expressing cells varied between 0.47±0.14 and 0.82±0.05. No differences between one point mutations and double point mutated integrin expressing cells were seen. These results indicate that the α2I domain binds to collagen type I by using a different mechanism than is uses to recognize collagen type IV.

5.3 Integrin binding differs between monomeric and fibrillar collagen (III)

Numerous studies have shown that collagen binding integrins can bind several collagen subtypes. In these experimental approaches, however, also the fibril forming collagens are presented for integrins as monomers. However, in tissues fibrillar collagens form large fibrils immediately after they are released by exocytosis from cells. In this study was tested whether the two major collagen receptor integrins, α1β1 and α2β1, can mediate cell adhesion also to collagen fibrils. For this, fibril formation by bovine skin collagen type I was initiated *in vitro* with soluble collagen preparation. This process was followed by measuring the turbidity (Fig. 1A/III) and by electron microscopy (Fig. 1B-D/III). At the starting point no fibrils were detected in solution (Fig. 1B/III). The saturation of fibrillogenesis reached the equilibrium state within 1 h with concentrations between 0.35 and 0.5 mg/ml (Fig. 1A/III). With lowest concentration (0.1 mg/ml) studied, no fibril formation was observed. The turbidity change measured was approximately proportional to the amount and/or diameter of fibrils formed in the reaction (not shown) as suggested in the first place by Wood and Keech (Wood & Keech, 1960, Wood, 1960a, and Wood, 1960b). In electron microcopy, both large, loosely packed bundles (Fig. 1C/III) and classical cross-striated fibrils were observed (Fig. 1D/III). In contrast to previous suggestion, the order of neutralization and warming of preparation had no effect on fibril morphology (not shown). The binding and immunoelectron microscopy experiments and spreading, collagen gel construction studies were carried out with monomeric and fibrillar preparations. Significant alterations in integrin recognition and binding during collagen fibrillogenesis were discovered. Furthermore, α1β1 and α2β1 integrins seem to have distinct functions in this process. For following results see Fig. 2/III, 3/III, 4/III, and 5/III.

5.3.1 The α1β1 and α2β1 integrins have lower binding affinity to fibrillar type I collagen than collagen monomers

The monomeric and fibrillar collagen type I binding of Europium labeled recombinant α1I/GST and α2I/GST domains were tested in solid-phase assay (Fig. 2/III). Binding at equilibrium was determined as a function of protein concentration, and the results were fitted to a Michaelis-Menten form equation to obtain suggestive kinetic parameters. The approximated K_d for α2I domain binding to monomeric collagen type I was about 10 nM (Fig. 2B/III). In contrast, the approximated K_d for α2I binding to fibrillar collagen was about 10-fold larger than for monomeric collagen (about 100 nM; Fig. 2B/III). In accordance with previous observations in publication I, the binding of α1I domain to monomeric collagen type I was weaker (about K_d 30 nM) than binding of the α2I recombinant (Fig. 2A/III). Importantly, α1I binding to fibrillar collagen type I was even weaker than to monomeric collagen (about 250 nM; Fig. 2A/III). The binding curve of αI domains did not only suggest that fibril formation may reduce the number of integrin binding sites, but also that the apparent K_d might decrease. Thus, it is possible that the changing conformation of collagen monomers during fibril formation reduces affinity of integrin receptor binding.

Due to the fact, that according to electron microscopy relatively large amount of fibrillar collagen was organized to loose bundles, the solid phase assay could not determine, whether the αI domains can actually bind to the tissue-type, tightly-packed, periodically electron dense fibrils. In order to observe binding selectively, the immunoelectron microscopy with was used to detect fibril-bound αI/GST proteins (Fig. 3A/III). For this, the protein A-gold - conjugate was linked to collagen bound fusion protein via GST antibody. Indicated by attached gold particles, the number of α2I domains (about 38) bound per μm of tightly packed fibril was higher than the number of bound α1I domains (about 20). This result is in accordance with results from solid phase binding assay. Furthermore, the images indicated that α2I domains can also bind to cross-striated fibrils (Fig. 3C/III), while α1I domains were mainly associated with loose fibrillar structures and only rarely seen on the surface of dense fibrils (Fig. 3B/III).

5.3.2 The α2β1 integrin can mediate interaction for both monomeric and fibrillar type I collagen

In the cell spreading assays the CHO-α2β1#12 cells spread slower on fibrillar collagen type I when compared to monomeric collagen, but fibrils could still support cell adhesion and spreading effectively (Fig. 4/III). The most cells were circular, and only a few fibroblast-like cells were seen. The spreading proportions after 120 min incubation were on monomeric and fibrillar collagen 64±7 and 76±9 %, respectively. The spreading on BSA as negative control was 7 %. These results confirmed conclusion based on α2I domain binding assay. In other words, despite a weaker interaction, when compared to monomeric collagen, α2β1 integrin can also mediate cell adhesion to fibrillar collagen. This was further functionally tested in 3-D collagen gel contraction assay. According to preliminary experiments the proportion of contraction was directly proportional to cell number (0-8x10^5 cells/ml) in the gel and varied between 15

and 33 % (not shown). The CHO-α2β1#12 cells ($3x10^5$ cells/ml) could mediate gel contraction unlike vector transfected control cells (Fig. 5A/III). After three days of cultivation, the surface area of gel was decreased with vector control and CHO-α2β1#12 cells by 12 and 44 %, respectively. This is in agreement with published data using fibroblast and osteosarcoma cells (Riikonen et al. 1995a, and Schiro et al. 1991). It was also confirmed that this collagen gel contraction requires high-affinity interaction with α2β1 integrin. For this double mutated CHO-α2D219N/D292Nβ1#2 cell line was used. It was shown in publication II, that this double mutation slows cell spreading on monomeric collagen type I, but does not prevent spreading completely and, furthermore, has no effect on spreading on collagen type IV. Here, this double substitution prevented collagen gel contraction indicating that α2β1 integrin with impaired capability to bind to collagen can not mediate collagen gel contraction. In this experiment, the contraction of wild-type and mutated cell line was 50 and 3 %, respectively (Fig. 5B/III).

5.3.3 Integrin α1β1 can not mediate high affinity cell adhesion to fibrillar collagen

In agreement with experiments presented in chapter 5.1., the CHO-α1β1#5 cells did not spread as fast as CHO-α2β1#12 cells on monomeric collagen type I (Fig. 4/III). Interestingly, the CHO-α1β1#5 cell spreading was even more modest on fibrillar collagen. The spreading proportion on monomeric and fibrillar collagen were 25±3 and 9±2 %, respectively. The spreading on BSA was 1 %. In addition, the α1β1#5 cells contracted collagen gels only a little bit better than control cells and clearly less than α2β1#12 cells (Fig. 5A/III). Thus, all these experiments support the conclusion that α1β1 integrin is not a primary receptor for fibrillar collagen. The suggestion that α1β1 integrin is a receptor for newly synthesized monomeric collagen is in agreement with previous observations naming α1β1 integrin as a negative feed-back regulator of collagen synthesis (Riikonen et al. 1995c, Langholz et al. 1995, and Gardner et al. 1999).

5.4 The cell adhesion domain of type XVII collagen is recognized by novel mechanism (IV)

Collagen type XVII (BP180) is a keratinocyte transmembrane protein that exists as full-length protein in hemidesmosomes and as a 120-kDa shed ectodomain in the extracellular matrix. The largest collagenous domain of collagen type XVII, COL15, has been described previously to support cell adhesion (Tasanen et al. 2000). In the work presented here, the integrin binding of both native and denaturated human recombinant COL15 was tested. The solid phase binding assay using recombinant α1I, α2I, α10I domains and the spreading experiments with α1β1 and α2β1 integrins expressing cells were carried out. The spreading studies with HaCat keratinocytes were carried by modifying the conditions with function blocking antibodies and synthetic collagen motifs mimicking peptides. To complete spreading data, the lateral migration assay were carried out. For experiments, the COL15 domain was expressed as a recombinant protein in human embryonic kidney cells. Data indicated that COL15 is not

recognized by collagen binding integrins, but binding is instead mediated by KGD-motifs in especially denatured collagen and RGD dependent integrins. For results see figures 1/IV, 2/IV, 3/IV, 4/IV, 5/IV and 6/IV, and Table I/IV.

5.4.1 Collagen receptor integrins α1β1 and α2β1 cannot recognize the native COL15 domain of type XVII collagen

The CHO-α1β1#5 and CHO-α2β1#12 cells were allowed to spread on rat type I, mouse type IV or the recombinant human COL15 domain. More CHO-α1β1#5 cells on collagen type mIV has spread (62±3 %) than those on collagen type rI (18±3 %; Fig. 1A/IV). In contrast, CHO-α2β1#12 cells spread faster on collagen type rI; 88±3 % *versus* 75±6 % on collagen type mIV (Fig. 1B/IV). These values are consistent with findings described in chapter 5.1. Both cell lines studied attached and spread on native COL15, although the spreading was much slower than on collagens rI and mIV. Only 14±6 % of CHO-α1β1#5 cells and 17±6 % of CHO-α2β1#12 cells had spread on triple helical COL15 (Fig. 1A-1B/IV). The spreading proportion of vector transfected cells was at the same level, suggesting that α1β1 and α2β1 dimers are involved in this process. This minor spreading response might be due to some other cell adhesion receptor expressed endogenously by CHO cells. Among spreading cells, both fibroblast and circular-like cell morphology was detected on all collagen types (not shown).

The binding of α1I/GST, α2I/GST, and α10I/MBP domains to native and heat denatured COL15 domain were tested in solid-phase assay (Fig. 1C-1E/IV). As a positive binding control collagen type rI for α1I/GST and α2I/GST domains and collagen type hII for α10I/MBP domain were used. The α1I/GST and α2I/GST domain binding to collagen type I was about $10x10^4$ and $15x10^4$ cps, respectively. The α10I/MBP domain binding of 35x103 cps to collagen type hII was detected. The binding of each αI domain tested to both native and denatured COL15 domain was comparable to the amount of bound recombinant detected from BSA wells. Importantly, this suggests that COL15 is one of the very few collagens that cannot be recognized by the collagen receptor integrins or their corresponding αI domains.

5.4.2 The COL15 is recognized after denaturation by α5β1 and αV integrins

In the second set of experiments, the human immortalized HaCaT keratinocytes were used in cell spreading assays on both native and denatured COL15 domain (Fig. 2A/IV). The most of the spreading was circular-like (not shown), the structural feature observed also by Tasanen et al. (2000). The total spreading on the native COL15 fragments at concentrations of 5 and 20 $\mu g/cm^2$ was 27±6 and 36±5 %, respectively. After denaturation the spreading was significantly increased. The cell spreading on denaturated COL15 preparation was 31±6 (5 $\mu g/cm^2$) and 53±2 % (20 $\mu g/cm^2$). When considering spreading behavior on native domain, it is notable that COL15 has a relatively low melting temperature (26 °C; Tasanen et al. 2000). Therefore, it is probable that at room temperature a portion of native collagen is partially denaturated and that only the denatured molecules mediate cell adhesion and spreading. The capability of HaCat keratinocytes to migrate laterally was tested using matrix precoated cell culture wells. Most cells plated on native COL15 detached during the first 2

days, and therefore no migration could be measured (not shown). Instead, the HaCaT cells stayed attached on denaturated COL15, and the cell migration during the 4 days was comparable with that on fibronectin (Fig. 3/IV). Within 2 days, the keratinocytes migrated 3.1±1.7 mm^2 on denatured COL15. In 4 days, the extents of migration on denatured COL15 and fibronectin were 10.8±1.8 and 10.3±1.5 mm^2, respectively. In accordance with spreading studies, the results from migration experiment indicate that denaturated COL15 domain support keratinocyte cell adhesion.

5.4.3 Collagen type XVII recognition and binding is mediated by KGD-motifs

Similarly to the nonactivated basal keratinocytes in skin, the HaCaT cells express the α2β1 integrins for collagen binding and α3β1 and α6β4 dimers for laminin binding. In addition, they express α5β1, αVβ1, and αVβ6 integrins for fibronectin and αVβ5 integrin for fibronectin. These dimers can usually be seen only in the activated skin keratinocytes (Koivisto et al. 1999, and Zambruno et al. 1995). Here, function blocking antibody against β1 integrin subunit could decrease the HaCat cell spreading on denaturated COL15 from 20±6 % to 5±2 % if compared to IgG containing antibody control (Fig. 4A/IV). The lower concentration, 1 μg/ml, had no effect on spreading. Inhibition detected is accordance with previous report by Tasanen et al. (2000) showed that β1 integrin subunit antibody can block HaCat cell adhesion to both native and denatured COL15. A cyclic RGD motif containing peptide (Koivunen et al. 1993), unlike a control peptide with an RGE motif, could effectively inhibit the cell spreading (Fig. 4B/IV). In this experiment, when no synthetic peptide was present, 30±9 % of HaCat cells spread on denatured COL15. In the presence of RGE peptide, 41±17 % of cells showed spreading like movement. When RGD peptide in concentration of 500 μM was added, the spreading decreased to 12±4 % of cells attached to the well surface. This proportion is equivalent to spreading on bovine serum albumin. Data presented here, indicate that the main cellular receptors for COL15 are the RGD dependent integrins containing β1 subunit. In the case of HaCat cell line, this suggests the involvement of α5β1 and αVβ1 dimers. This is supported by the fact that functional blocking αV subunit antibody could block the HaCat cell adhesion to COL 15 (Fig. 5A/IV) in a concentration dependent manner. In the presence of highest αV antibody concentration (5 μg/ml) used, the spreading was decreased from 44±9 % to 25±5 %. Further, the α2 and α3 subunit antibodies had no effect on spreading (not shown). The involvement of α5β1 integrin was confirmed with functional α5 subunit antibody (Fig. 5B/IV). The spreading with α5 antibody was inhibited from 53±2 % to 25±5 %. The presence of β1 antibody inhibited spreading about 70 %, indicated by proportion of 16±3 %. When α5 and αV antibodies were used together to inhibit spreading, they were slightly more effective (21±7 %) than anti-α5 alone (Fig. 5B/IV), but not more effective than β1 antibody. These results suggesting that α5β1 and αVβ1 dimers are involved in COL15 binding, were confirmed in separate set of experiments with native collagenous fragment. On native COL15, the antibody combination stated above inhibited cell spreading from 31±6 to 8±6 %. This suggests that HaCaT cells, despite the slower spreading rate, use the same integrins to spread both on native and denatured COL15 domain.

The collagen type XVII does not contain any known integrin binding sequences (CAC00589). The data described above suggest strongly that despite the fact that COL15 domain contains no RGD motifs, the integrin recognition of this collagenous domain is related to laminin and fibronectin binding. Instead, each of three α-chain in COL15 homotrimer contains four highly RGD-related KGD motifs. This repeat is also found in snake venom proteins (Scarborough et al. 1991, and Oshikawa & Terada, 1999) which are able to interact with RGD-dependent integrins. This suggests that COL15 could use the same mechanism. For this, the entire sequence of COL15 domain as 22 overlapping peptides (12-14 amino acids) was studied competitively in cell spreading assays (Table I/IV). To begin with, the ability of peptides to inhibit cell spreading on denatured COL15 domain was tested with concentration of 0.7 mM (Table I/IV). The five peptides inhibiting spreading over 90 % were tested further with concentrations of 0.1 and 0.01 mM (Fig. 6A/IV). The inhibition with these peptide concentrations varied between 19 and 93 %. Each of three best inhibiting (> 60 %) peptides contained one KGD motif in each. The peptide (LRGEVGLPGVKKGDK) showing the strongest inhibition was mutated to have a glutamate residue instead of aspartate (...KGE...). In an independent assay, this substitution increased spreading from 18±2 % to 44±8 % if compared to spreading in the presence KGD-peptide (Fig. 6B/IV). The spreading without peptides on COL15 was 54±11 %. The collagen type XVII RGD-dependent recognition mechanism is unique in collagen family reported so far.

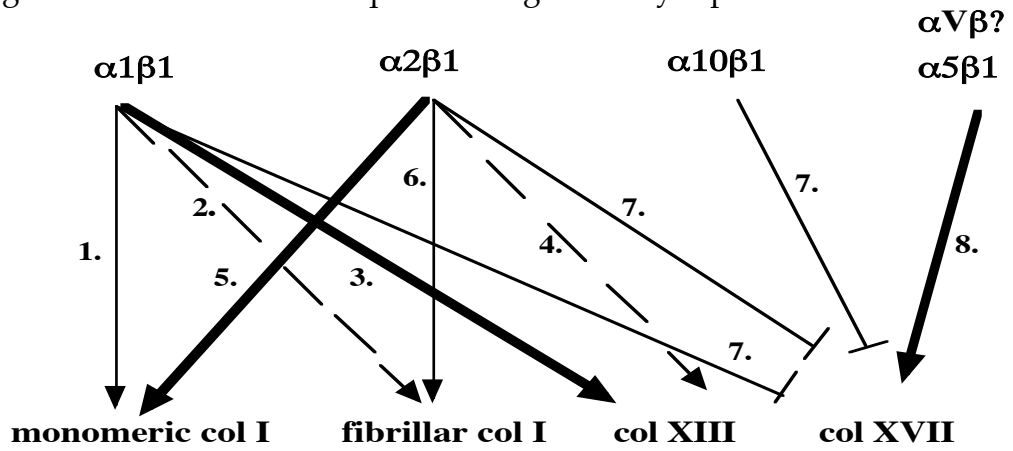


FIGURE 5 The summmerized results as conclusions from this thesis concerning ligand specifity of collagen binding α1β1, α2β1, and α10β1 integrins. The α1β1 integrin recognizes monomeric collagen type I (1.), but only weakly collagen type I as fibrils (2.). The α1β1 dimer binding of transmembrane collagen type XIII is effective (3.), unlike weak integrin α2β1 dimer binding (4.). The α2β1 integrin recognizes and binds monomeric collagen type I effectively (5.) and has also affinity to collagen type I as fibrils (6.). The tested collagen binding integrins does not bind the collagenous domain, COL15, of collagen type XVII (7.). This domain is instead recognized by αVβ and α5β1 integrins (8.).

6 DISCUSSION

6.1 The central methods in current study (I-IV)

As reviewed before, the collagen-integrin interaction is known to be based on the molecular recognition of collagens by αI domains which are located in the extracellular part of α1, α2, α10, and α11 collagen binding integrin subunits (Kamata & Takada 1994, and Kern et al. 1994, Camper et al. 1998, and Velling et al. 1999). In addition it is known that these independently folding structures retain many of the functional properties of the parent integrin, and therefore are used generally as models for studying integrin functions (for example see Muchowski et al. 1994 and Tuckwell et al. 1995). Based on this knowledge, the α1I and α2I domains as classical collagen binding structures were produced as recombinants and solid phase binding assays with different immobilized collagen subtype monomers were carried out in the presence of Mg^{2+}. In publications I and II the GST fusion partner was removed enzymatically and purified recombinant α1I and α2I domains were labeled directly with Europium. In these experiments the saturation of collagen binding was not achieved despite the concentration of αI^{Eu} was increased up to the maximal detection limit of fluorometry. For this reason binding kinetics including K_d values could not estimated. In manuscript III and publication IV GST or MBP fusion partners were retained intact. The binding was measured via Europium labeled antibodies against the fusion partners. In this method normal kinetics and saturation of binding was achieved. It is probaply that when αI domains are labeled directly, the isothiocyanate group of labeling reagent reacts with the free amino group of recombinant in collagen recognition and binding area, and therefore disturbs the αI domain-collagen interaction.

When α1 and α2 integrin subunit transfected chinese hamster ovarian cells adhere to precoated collagen surfaces, no significant differences in the total amount of cells adhered to BSA as negative control and to different collagen types were observed. This indicates that adhesion studies based on cell staining and fluorometric measurement of attached color are no sensitive. Based on this, the rate of cell spreading behavior was chosen as an indicator of integrin function.

The results of both spreading and αI domain based solid phase experiments are dependent on collagen preparation utilized. All fibrillar collagens and collagen type IV used were commercially obtained and importantly non-pepsin purified. However, some degradation in collagen IV preparation was observed occasionally as indicated by vector control cell spreading. This behavior is dependent on exposed binding sites for non-collagen binding integrins. In publication III for fibrillogenesis collagen type I was used, which was prepared from pepsin-treated bovine dermis. It contains 5 % collagen type III as addendum. However, as reviewed, heterotypic fibrils composed of both collagen type I and III occur in nature (Myllyharju & Kivirikko 2001). This preparation produced both cross-striated collagen type fibrils and loose fibrillar structures. Loose collagen bundles are probably due to partly damaged telopeptides during purification procedure even though their role in collagen fibrillogenesis in under discussion.

6.2 Fibril and network forming collagens are recognized by different mechanisms (I, II)

Several cell types both in adults and fetal tissues, can express all four collagen receptors concomitantly at least at the mRNA level (unpublished data from this project). In addition, the integrin expression pattern of cells changes during different physiological and pathological conditions regulated by cytokines and growth factors (Adams & Watt 1990, Korhonen et al. 1990, Larjava et al. 1993, and Nikkari et al. 1993). This raises the question about requirement and possible separate functional differences between the collagen binding integrins. At least α1β1 and α2β1 integrins seem to have separate signaling functions induced by collagen binding (Ivaska et al. 1999b). These differences can be seen among other areas but in regulation of ERK signaling pathway (Wary et al. 1996, Wary et al. 1996, and Ivaska et al. 1999b).

The molecular mechanism of collagen recognition and binding by integrins are mostly unknown in molecular details despite the importance of this data in understanding the cell-matrix interaction. The binding sites for α1β1 and α2β1 integrins have been localized to native collagens; i.e. triple helical domains in collagen proteins (Eble et al. 1992, and Gullberg et al. 1992). However, it is known that collagenous triple helix is not sufficient alone for integrin binding (Tuckwell et al. 1996). Further, some collagens among other matrix proteins has RGD motifs in their α chains but they seem to be recognized only in denatured or degraded collagen by fibronectin and vitronectin receptors (Ivaska et al. 1998). A linear DGEA peptide sequence derived from collagen type I α(I) chain has been reported to block both collagen- and laminin-integrin interaction (Staatz et al. 1991). However, this result was not satisfactorily repeated (Cardadelli et al. 1992, Pfäff et al. 1993, and Tuckwell et al. 1995). Numerous studies have suggested the role of aspartic acid, glutamic acid, and arginine residues in collagen binding by both α1β1 and α2β1 integrins (Eble et al. 1993, Emsley et al. 1997, Nolte et al. 1999, and Kamata et al. 1999). The cyclic or triple helical peptides containing charged amino acid repeats like arginine-lysine-lysine (RKK; Ivaska et al. 1999a, and Pentikäinen et al. 1999) derived from snake venom toxin, jararhagin, can block competitively ligand-receptor interaction at least between collagen types I, IV

and laminin-1 and integrin α2I domain. However, it is notable that neither collagen type I or IV α chains contain this motif. It is more likely, that this epitope is formed by amino acids from two or even three α chains in collagen monomer surface. Knight et al. (1998) pointed out the importance of GER motif in native collagen type I recognition by α2β1 integrin dimer. In further studies, the sequence was specified to be GFP$^{(OH)}$GER for both α1β1 and α2β1 integrins (Knight et al. 2000). In addition, Xu et al. (2000) reported that the chemically related GLP$^{(OH)}$GER motif is recognized by collagen binding integrins. However as obvious due to diversity, these motifs cannot determine the specificity for integrin receptor recognition. Further, the experimental data presented in publication I indicated that presence of these motifs in collagen α chains does not predict the collagen binding by integrin. This indicates the existence of still an unknown additional and necessarily more specific recognition motifs for collagen binding integrins.

6.2.1 Difference between collagen types I and IV

From an expression pattern and signaling point of view, a tempting possibility is that α1β1 and α2β1 integrins can bind selectively to some of the almost 30 known collagen types. However, previous reports have not supported this idea. Blood platelets were shown to bind to collagen types I-VIII and this interaction could be blocked with α2 integrin subunit function blocking antibody (Saelman et al. 1994). In another study, interaction also with collagen type XI was shown (Tuckwell et al. 1996). These results indicate the existence of α2β1 dimer as a wide-spectrum collagen binding receptor. As well, the interaction of α1β1 or α1I domain with collagens I-VI has been reported (Pfaff et al. 1993, Ruggiero et al. 1994, Kamata et al. 1999, and Riikonen et al. 1995b). Only one difference in binding has been reported so far. The α1β1 integrin dimer favors basement membrane associated network forming collagen type IV, whereas α2β1 integrin dimers seem to have better affinity for fibril-forming collagen type I (Kern et al. 1993, Calderwood et al. 1997, and Kern & Marcantonio, 1998). In this thesis, the phenomenon was clearly seen in spreading studies with cells expressing either α1β1 or α2β1 integrins. The α1β1 expressing cells spread faster on collagen type IV and the α2β1 cells on collagen type I than *vice versa*. However, the difference in solid phase binding assay based on direct Europium labeled recombinant αI domain was small or could not be detected at all. The collagen type I monomer contains typically four GLPGER motifs and two GFPGER motifs as known potential binding sites for integrins. Correspondingly, type IV monomer contains two GFPGER motifs. This difference offers on interesting explanation for binding preferences. However, it is notable that both collagen types stated are heterotrimers and the chain composition varies in different trimer isoforms though affecting the number of binding sites. From the binding experiments point of view, it seems that primary αI domain recognition and binding do not determine alone the affinity of binding. Possibly, the ligand interaction with β1 subunit in integrin dimer is needed in addition. At least the mechanism of α2I domain binding between collagen type I and IV seems to be different.

6.2.2 The molecular bases on integrin-collagen interaction

One possibility is that metal ion, Mg^{2+}, present in all αI domains at the MIDAS site (Lee et al. 1995, Emsley et al. 1997, Rich et al. 1999, Nolte et al. 1999,

Salminen et al. 1999, and Qu & Leahy, 1995) directly mediates collagen binding (Emsley et al. 1997). The exact role of metal is under discussion, but most models do locate collagen binding site close to the MIDAS. In addition it has been suggested, that some charged amino acids surrounding the MIDAS might have an important role in recognition of collagen type I. In previous reports, however, Asp219 -> Ala, Glu256 -> Ala, or Asp292 -> Ala, point mutations did not show effect on CHO cell attachment on collagen (Kamata & Takada 1994), unlike mutations to residues directly participating in Mg^{2+} binding (Kamata et al. 1994). Despite this, the possibility that Asp219 may participate in collagen binding was suggested later by molecular modeling data (Emsley et al. 1997). In addition, we have recently published indirect evidence that Asp259 and Asp292 may participate in collagen recognition by suggesting their interaction with RKK peptide which inhibits the collagen type I binding (Pentikäinen et al. 1999). In our study presented in publication II α2I domain binding to collagen type I was monitored in real time biosensor system. The data was supplemented with solid phase binding studies with α2I domains and cell spreading studies. The results indicate that residues Asp219, Asp259, Asp292, and Glu299 are important for collagen type I binding of α2I domain. However, any of these mutations could not completely prevent the collagen binding. This suggests that several amino acids in the MIDAS area make concomitant effect and might have even a co-operational contribution in this phenomenon. This might explain why Kamata & Takada (1994) did not observe any effect with mutations introduced to collagen in CHO cells. We transfected CHO cell clones to introduce Asp219 and Asp292 single mutations to full-length α2 integrin subunit cDNA and carried out cell spreading studies. In agreement with negative results mentioned above, it was not able to see differences when compared cells expressing wild-type α2 integrin subunit. However, the simultaneous contribution of Asp219 and Asp292 could be confirmed in spreading experiments in which CHO cell clone carrying both amino acid mutations were used at the same time. This is first direct evidence that collagen binding site is located near the MIDAS and the amino acids in close relation to this structure, but none interacts directly with Mg^{2+} are involved in collagen recognition.

Interestingly, despite the fact that the spreading of double mutation carrying cells was decreased on collagen type I, this set of mutations had no effect on collagen type IV recognition and binding. The double mutated cells could spread on collagen type IV with similar efficiency as the wild-type α2β1 expressing cells. This indicates, that fibrillar collagen type I and network like structures forming collagen type IV are recognized by distinct mechanism. As different to each other, in α1I domain, instead of negatively charged Asp219 in α2I, corresponding residue is positively charged Arg218. The idea concerning the fact that α1I domain binds better to collagen type IV could be due to one single amino acid residue was tested by making the mutation Asp219 -> Arg for α2I binding studies. However, the mutant preferred still collagen type I over type IV indicating that this hypothesis was wrong.

In addition to critical residues mentioned above, it is probable that in the putative collagen binding surface of α1I and α2I domains, the αC helix participates to collagen binding. However, its participation in collagen recognition has never been shown. This structure is also found in α10I and α11I domains, and it is unique to collagen binding integrins found this far (Emsley et al. 1997, Rich et al. 1999, Nolte et al. 1999, and Salminen et al. 1999). The major

difference in the supposed ligand binding surfaces of α1I and α2I domains, is in the bulky αC helix of α2I domain determined by Tyr285 and Asp289 oriented to towards the supposed binding groove. In α1I domain these residues are correspondingly sterically smaller than Ser284 and Gly288. The importance of αC helix was tested by deleting central residues from this structure in α2I domain. According to molecular modeling, this modification did not cause any drastic structural changes in α2I domain structure, and according to experimental data the interference with Mg^{2+} orienting site was not observed. This was probably due to the fact that αC helix is a "separate loop" on the surface of the αI domains. The biosensor experiments showed that this additional loop is important for collagen type I binding. The apparent K_d of wild-type binding was to close to values reported earlier and measured in the presence of Mn^{2+} (Calderwood et al. 1997). The approximated K_d of ΔαCα2I was about five-fold higher than the K_d of wild-type. These changes in kinetics can be explained that after helix deletion more space is available for the binding of collagen type I in the binding groove of α2I domain. In the absence of αC helix, the specificity of collagen type I binding is decreased, and the association and dissociation of the ligand occurs more easily. In addition, the ΔαCα2I binding to collagen types I and IV showed that binding profile is different when compared with the profile of the wild-type α2I domain. The αC helix deletion αI domain seemed to bind better to collagen type I than to collagen type IV; the binding profile resembling that has been presented for α1I domain (Calderwood et al. 1997). The collagen type IV binding was saturating at higher coating concentrations than the collagen type I binding. However, as described before, according to the solid phase binding assays, the difference between collagen types I and IV binding of α1I domain was very small. Still it is possible, that the bulky αC helix in α2I domain narrows the collagen binding groove and may therefore disturb the collagen type IV binding, which contains in addition noncollagenous sequences between more compact triple helical domains. Instead, the collagen type I monomer is sterically more compactly packed because of non-interrupted collagenous section.

6.2.3 The existence of different binding mechanisms among fibrillar collagens

All fibrillar collagens as monomers, tested in addition to type I, i.e. types II, III, and V are recognized both by α1I and α2I domains in solid phase binding studies. This finding is accordance with previous reports (Pfaff et al. 1993, Ruggiero et al. 1994, Riikonen et al. 1995b, and Kamata et al. 1999). Interestingly human collagen type III and V α chain peptides do not contain known integrin binding motifs. Despite this fact, they still mediate also cell spreading of both α1β1 and α2β1 expressing cells. This indicates the existence of an unknown collagenous recognition and binding mechanism. On the other hand human collagen type II α chains do contain both GLPGER and GFPGER motifs, but these repeats seem to be inactive in this context because collagen type II monomers do not enhance the spreading of α1β1 integrin expressing cells. In accordance with previous studies (Calderwood et al. 1997), the α1I the could effectively bind to collagen types II tested from different origin. The α2β1 integrin subunit expressing cells spread in accordance with results from binding studies. The function blocking α1 and α2 integrin subunit antibodies can decrease the spreading of most cell types on collagen (Pfaff et al. 1993,

Saelman et al. 1994, Ruggiero et al. 1994, and Riikonen et al. 1995b). However, the participation of some other than collagen binding receptor in the cell-matrix interaction can not be excluded. In the case collagen type II it was evident that α1I domain recognition does not lead to cell spreading. In agreement with this, we have previously shown that α1β1 dimer function blocking antibody cannot affect HeLa cell attachment to collagen type II, despite the fact that it can inhibit binding to collagen type I and almost completely block attachment to collagen type IV (Riikonen et al. 1995b). It is possibly that α1β1 integrin needs an assisting receptor for efficient collagen type II binding. For example, cell surface proteoglycans, such as syndecans (Elenius et al. 1990) and CD44 (Knutson et al. 1996), can bind to collagens. During the spreading and migration on collagens, the integrins and proteoglycans may co-operate (Knutson et al. 1996). This effect was not possible seen because CHO cells used in this study might lack this additional receptor needed. It is also notable, that cells were detached by quite intensive trypsination, and some membrane structures, like syndecans, are very sensitive for endopeptidase activity.

6.3 Collagen recognition is altered during fibrillogenesis (III)

All the binding and spreading experiments presented this far have dealt with collagens as monomers, despite the fact that fibril and network forming collagens do not stably exist as monomers *in vivo*. Instead, they are spontaneously assembled into supramolecular structures after secretion by exocytosis. Until now, it is not known if collagen receptor integrins can bind directly to the tightly packed collagen fibrils. Furthermore, the situation *in vivo* is more complicated because fibrils are often covered by glycoproteins and proteoglycans, including fibronectin, decorin and FACIT collagens (for review and references therein see Prockop & Kivirikko, 1995). In this study within publication III it was tested to see if α1I and α2I domains as recombinant proteins and either α1β1 and α2β1 integrin expressing CHO cells can interact with bovine skin fibrillar collagen type I. The binding data was supplemented with immunoelectron microscopy. As described in results above and previous publications (Lee et al. 1995, and Kamata & Takada 1994), both α1I and α2I domains mediate the adhesion to monomeric collagen type I. Binding data indicate that α2I protein can bind to fibrillar collagen type I with relatively low apparent K_d (about 100 nM). However, this interaction is much stronger than corresponding binding of α1I domain (K_d about 250 nM). The findings in immunoelectron microscopy are in agreement with this. In addition, α2I domain can recognize both loose collagen bundles and tightly packed cross-striated fibrils. As described before in this study (publication I) and other reports, α1I domain can bind to monomers of fibril-forming collagen types I, II, III, and V, but still it seems to favor basement membrane collagen type IV. According to these, the α2I domain has opposite ligand binding preferences (Kern et al. 1993, Calderwood et al. 1997, and Tulla et al. 2001). Some preliminary data suggests that α10I domain binding resembles more α1I binding (Tulla et al. 2001). In this respect, it seems that α10I is not a receptor for collagen in fibrils. This indicates further, that integrin α2β1 dimer is the main receptor for fibrillar collagen. However, the importance in fibril binding of α11β1 integrin remains to be shown. The negative role in α1β1 integrin

adhesion to fibrillar collagen is supported by the fact that this dimer cannot effectively initiate the construction of floating collagen gels as in response to collagen binding integrin signaling. It has been suggested, that also α1β1 dimer, and αVβ3 integrin as receptor for fibronectin and vitronectin, could also mediate collagen type I gel contraction (Gotwals et al. 1996, Racine-Samson et al. 1997, and Cooke et al. 2000). However, Ivaska et al. (1999) have shown that integrin α2 subunit containing the cytoplasmic domain of α1 subunit can not mediate collagen type I gel contraction (Ivaska et al. 1999a). The data presented indicates that α1 integrin subunit activity of intracellular domain, i.e. integrin mediated outside-in signaling, is not sufficient to induce cell spreading and migration. However, it is notable that the response activated by integrin binding might vary between cell types due to the ligand introduced for cells and ligand concentration available. The importance of intact α2β1 integrin dimer was also demonstrated by result that the double mutated α2β1 integrins (α2D219/D292Nβ1) could not mediate the collagen type I gel construction. The results concerning the binding preferences fit very well to signaling functions reported for α1β1 and α2β1 integrins in cell regulation. The α1β1 dimer is a receptor for monomeric type I only and, as reviewed before, acts as a negative feed back regulator of collagen synthesis. In contrast, the α2β1 dimer can bind both monomeric and fibrillar collagen, and as a response increase the matrix turn-over and remodeling (Riikonen et al. 1995, Langholtz et al. 1995, and Gardner et al. 1999).

The formation of collagen fibrils is entropy driven, spontaneous reaction *in vitro*. However, may cells have several ways to control this reaction *in vivo*. Extracellular matrix proteins may initiate the formation of fibrils, and the additional, minor types collagen monomers have a certain role in heterotypic fibril formation at least *in vitro*. Other proteins, like thrombospondin and decorin, seem to regulate the size of fibrils from its part (Danielson et al. 1997, and Kyriakides et al. 1998). The better affinity of α1β1 and α2β1 dimers for monomeric collagen indicates also the integrin participation of it in fibrillogenesis. It is tempting to hypothesize that an integrin might collect newly synthesized collagen close to cell surface, and though target the fibril formation to the pericellular area. After fibril formation, the affinity decreases and monomeric collagen molecules can replace the bound fibrils from receptors.

6.4 Transmembrane collagens from the integrin point of view (I, IV)

The subgroup of transmembrane collagen contains two characteristically plasma membrane situated members i.e. collagen type XIII and XVII. Their interactions with other molecules, and over all, their biological function are under investigation. In addition to their unique transmembrane domain among the collagen family, they are further released from cell surface to the extracellular matrix (Schäcke et al. 1998, Hirako et al. 1998, and Snellman et al. 2000b). This allows them to function as matrix proteins contributing to cell adhesion and migration. However, structurally type XIII and XVII molecules are different. The collagen type XIII has three relatively large collagenous domain, whereas type XVII has only one large and 14 short collagenous domains. These new members in the collagen family show new properties

suggesting that they are not just structural proteins in connective tissues but that they have more complex functions.

6.4.1 A novel difference between α1β1 and α2β1 integrins

In solid phase binding experiments, the α1I domain bound effectively to recombinant collagen type XIII if compared to the marginal α2I domain binding. The difference was even bigger than indicated by very sensitive spreading studies between collagen types I and IV. This is the first demonstration that α1I and α2I domain can selectively recognize some of collagen types and therefore indicate the separate function of α1 and α2 subunits. The finding was further confirmed in spreading studies. The α1β1 dimer expressing CHO cells spread effectively on collagen type XIII coated surface unlike α2β1 cells. This finding might provide an interesting possibility for detailed structural studies which can shed light to α1β1 dimer function. It can be speculated, that collagen type XIII in transmembrane position might mediate the cell adhesion of neighboring cells to each other. However, more likely, the shed, soluble form supports the cell-matrix interaction instead of cell-cell interaction. When comparing the α1β1 cell spreading on collagen type XIII and on type IV, an interesting difference was observed. In addition to typical fibroblast-like cells, circular cells having extensive plasma membrane ruffles were detected. This morphological transition was observed throughout the collagen types and concentrations tested, but this difference was particularly detectable in the case of collagen type XIII. Almost all the cells were circular-like on type XIII surface in contrast to collagen type IV. This suggests differences in the collagen binding mechanisms and further in outside-in signaling responses initiated by ligand-receptor interaction. The possible role of small GTP-ases, i.e. Cdc42, Rac, and Rho is quite obvious. These effectors have been shown to interact among others with integrins and to regulate the morphology, moving, and the phenotype of cells. When activated Rho family enzymes induce actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression and membrane trafficking (Ridley et al. 1999 and Schwartz & Shattil 2000). The cellular response of GTP-ase activity is supposed to be dependent on cell type and origin, the type of ligand bound and the ligand concentration available for cellular receptors (Schmitz et al. 2000). Based on this, the collagen types I and IV, inducing both spreading morphologies, were preacoated on different concentrations, and α1β1 and α2β1 cells were allowed to spread on these surfaces. The occurrence of fibroblast-like, both α1β1 and α2β1 cells, was directly proportional to collagen type I concentration but only few circular-like cells were detected. On collagen type IV, a bell-shaped dependence of spreading and spreading morphology as function of substrate concentration were seen with both cell lines. Physiologically, the fibroblast-like morphology may present the migrating cells, while the circular-like cells may not be able to move. In addition, the difference of cell morphology may also be due to the number of integrin-binding sites in one collagen monomer or the action of an assisting receptors. Interestingly, as well as the III and V collagen molecules as fibril forming collagens, the collagen type XIII with homotrimeric α chains do not contain any known integrin binding motifs.

6.4.2 Collagen type XVII as an unique collagen family member

The largest collagenous domain of human collagen type XVII, called COL15, has been reported to support keratinocyte adhesion. This interaction was blocked by function blocking β1 integrin subunit antibody (Tasanen et al. 2000). However, according to solid phase binding and spreading studies collagen binding integrins tested, α1β1, α2β1 and α10β1 dimers do not seem to mediate this binding neither on native nor denaturated COL15 domain. Until now, all the collagens tested have been shown to be able to bind at least one of these receptors, although, as reviewed before, the collagenous triple-helix alone is not sufficient to support cell adhesion (Tuckwell et al. 1996). Interestingly, as well as collagen types III, V and XIII tested, the COL15 domain does not contain any known integrin binding motifs. As described, the type XVII extracellular part is shed from the cell surface proteolytically (Schäcke et al. 1998, and Hirako et al. 1998) which may lead to denaturation of the collagenous structure. The 50 % of native ectodomain structure is lost at 41.5 °C under neutral pH (Schäcke et al. 1998). *In vitro*, the recombinant COL15 domain, when it is not a part of entire type XVII ectodomain, will denature at body temperature (Tm 26.5 °C; Tasanen et al. 2000). Importantly, the denaturation seems to make COL15 domain as cell adhesion ligand by exhibiting the integrin binding sites. According to solid phase binding experiments with integrin function blocking antibodies, the recognition seems to be mediated by a fibronectin receptor, a5b1 integrin, and by fibronectin/vitronectin receptors containing the aV subunit, especially aVb1 integrin dimer. The a5b1 integrin is solely RGD dependent receptor. The aV subunit in less selective. It can recognize in addition to a RGD containing ligands, several other proteins. These include metalloproteinase-2 (Brook et al. 1998), a disintegrin ADAM 23 (Koivisto et al. 1999), the fibrinogen g-chain (Yokoyama et al. 2000), and the COOH-terminal domain of tenascin C (Yokoyama et al. 2000). However, none of these has any similarity to COL15. Snake venom derived proteins, like barbourin (Scarborough et al. 1991) and ussuristatin 2 (Oshigawa & Terada, 1999), contains chemically RGD-like motifs, namely KGD repeats. This motif, however, binds with much better affinity to the platelet fibrinogen receptor, αIIbβ3 dimer, than to α5β1 integrin (Scarborough et al. 1991). The αV recognize the KGD site with even lower affinity than α5β1 integrin (Scarborough et al. 1991). The COL15 domain has four KGD sites in each of the three a chains and these repeats seem to form a molecular base for its recognition by keratinocyte integrins. The RGD containing peptides can competitively inhibit this interaction. Further, three COL15 derived peptides with KGD motif could inhibit the binding. After, the KGD was mutated to KGE, the peptide activity was abolished. Although, the a5 and aV subunits containing integrin affinity for KGD motif has been reported to be relatively low (Scarborough et al. 1991, and Yokoyama et al. 2000), it still might be sufficient for cell adhesion (Yokoyama et al. 2000) and promote cell migration. It is probable that the amino acids following the KGD repeats have some influence on receptor specificity (Scarborough et al. 1991). In accordance with this, all collagen type XVII derived KGD containing peptides do not inhibit the cell spreading on COL15 surface. It has been suggested that KGDW sequence may promote high affinity binding for aIIbb3 receptor, a whereas KGDD may be a better ligand for a5b1 integrin dimer (Scarborough et al. 1991). Here, the two most strongly binding peptides carried arginine or

lysine as the fourth amino acid. The peptides with KGDM and KGDQ motifs were much less efficient. It is also possible that other amino acids close to the KGD motif within the peptide may modify this binding. The α5β1 integrin mediated cell binding to the fibronectin require in addition to RGD motif from repeat 10, so-called "synergy sequence" from repeat 9 (Mould et al. 1997). The existence of similar mechanism in COL15 recognition can not be excluded and due to shortness of synthetic peptides this phenomenon is not seen.

7 SUMMARY

Major increase in the complexity occurred during animal evolution at the transition from the unicellular protozoan to a multicellular metazoan. The origin of these animals was accompanied by appearance of extracellular matrix (ECM) and extracellular matrix binding proteins. Possibly one of the most important molecular inventions was the development of collagens and collagen binding integrins which are common to all nucleated cells. Together they affect actively and regulate the attachment, proliferation, migration, differentiation, and metabolism of surrounding cells during phenomena such as development and morphogenesis. Classical integrin-type collagen receptors, α1β1 and α2β1 integrins, are structurally very similar and represent the same evolutionary branch in integrin family. However, cells can concomitantly express both receptors and it has been further shown that these collagen binding integrins have distinct signaling functions, and their binding to collagen may lead to opposite cellular responses.

Here, CHO cells, which lack endogenous collagen receptors, were transfected with either α1 or α2 integrin subunit cDNA in order to express α1β1 and α2β1 dimers. Cells were allowed to attach to various immobilized collagen types, and as an indication of active integrin function in progression of cell spreading was observed. Collagen binding integrins recognize and bind collagens with specific inserted domains, called αI domains. The α1I and α2I domains were produced as recombinant proteins, labeled with Europium and used in a sensitive solid-phase assay on precoated collagen surfaces. When necessary, data from these two keystone methods was supplemented by immunoelectron microscopy, biosensor experiments, molecular modeling, collagen gel contraction, and lateral migration assays. Studies were carried out either with wild-type or mutated structures.

The cells expressing α1β1 integrin could spread on collagen types I, III, IV, and V but not on type II, while α2β1 integrin could mediate cell spreading on collagen types I-V. Type XIII is a transmembrane collagen and its interaction with the integrins has not been previously studied. CHO-α1β1 cells could spread on human recombinant collagen type XIII, unlike CHO- α2β1 cells. In accordance with this finding, the α1I domain, unlike α2I domain, could attach to collagen type XIII. The results indicate, that α1β1 and α2β1 have different ligand binding specificity. Distinct recognition of different collagen subtypes by

the αI domains can partially explain the differences seen in cell spreading. However, despite the fact that CHO- α1β1 cells could not spread on collagen type II, α1I domain could bind to this collagen type which indicates an important role of β1 subunit or an assisting receptor in collagen binding.

A common structural feature in the collagen binding αI domains is the presence of an extra helix, named helix αC. However, its participation in collagen binding has not been shown. Here, helix αC was deleted in the α2I domain and the function of the resultant recombinant protein was tested by using a real-time biosensor. The deletion mutant had reduced affinity for collagen type I (430±90 nM) when compared to wild-type α2I domain (90±30 nM), indicating both the importance of helix αC in collagen type I binding and that collagen binding surface in α2I domain is located near the metal ion binding site (MIDAS). Previous studies have suggested that the charged amino acid residues, surrounding the MIDAS but not interacting with Mg^{2+}, may play an important role in the recognition of collagen type I. Direct evidence indicating the participation of these residues in collagen recognition has been missing. To test this idea we produced a set of recombinant α2I domains with different substitution mutations, namely D219A, D219N, D219R, E256Q, D259N, D292N, and E299Q. Mutations in amino acids Asp219, Asp259, Asp292 and Glu299 resulted in weakened affinity for collagen type I. When α2 D219N and D292N mutations were introduced separately into α2β1 integrin expressed CHO cells, no alterations in the cell spreading on collagen type I were detected. However, CHO cells expressing double mutated α2D219N/D292N integrin showed remarkably slower spreading on collagen type I, while spreading on collagen type IV was not affected. The data indicate, that α2I domain binds to collagen type I with a different mechanism than to collagen type IV.

Numerous studies have shown their binding to various collagen subtypes, including fibril-forming collagens. However, in tissues these collagens form large fibrils immediately after they have been released from cells. Here we have tested whether the two major collagen receptor integrins, α1β1 and α2β1, can mediate cell adhesion to collagen fibrils. A solid phase binding assay was developed to estimate the binding constants of their collagen binding αI domains. Integrin α2I domain bound to monomeric bovine skin collagen with a $K_d \approx 10$ nM and to the corresponding fibrils with a $K_d \approx 100$ nM. The data indicates that fibril formation affects the binding constant and at the same time reduces the number of putative binding sites available to the integrins. Immunoelectron microscopy confirmed the binding of α2I domains to tightly packed, cross-striated fibrils. Despite its weaker binding, integrin α2β1 could still mediate cell adhesion and spreading on fibrillar collagen. CHO cells transfected to express α2β1 as their only collagen receptor could also mediate the contraction of floating collagen gels. Another collagen receptor, α1β1, was a significantly weaker receptor for fibrillar collagen. CHO-α1β1 cells could neither adequately spread on collagen fibrils nor mediate the contraction of collagen gels. This is explained by the weak binding of α1I domain to fibrillar collagen ($K_d \approx 250$ nM). These findings indicate that α2β1 integrin is a functional cellular receptor for collagen fibrils, while α1β1 integrin may only bind collagen monomers effectively. These observations complement the previous observations that α1β1 and α2β1 integrin collagen receptors have distinct signaling functions and suggest that they participate in pericellular collagen fibrillogenesis.

Collagen type XVII (BP 180) is a keratinocyte transmembrane protein

which exists as a full-length protein in hemidesmosomes and as a 120 kDa shed ectodomain in the extracellular matrix. The largest collagenous domain of collagen type XVII, COL15, has been previously described as a cell adhesion domain. In the present work, the integrin binding of triple helical, recombinant human COL15 was tested. Solid-phase binding and cell spreading assays showed that, unlike other collagens, COL15 was not recognized by the collagen receptors. In addition to α1I and α2I domains, α10I was tested. Denaturation of COL15 domain increased the spreading of human HaCaT keratinocytes, which could migrate on denatured COL15 domain as effectively as on fibronectin. Spreading of HaCaT cells on COL15 domain was mediated by α5β1 and αVβ1 integrins and it could be blocked by RGD peptides. The collagen α-chains in the COL15 domain do not contain RGD motifs but instead, twelve closely related KGD motifs, four in each of the three α-chains. Twenty-two overlapping, synthetic peptides corresponding to the entire COL15 domain were tested; three peptides, all containing the KGD-motif, inhibited the spreading of HaCaT cells on denatured COL15 domain. Furthermore, this effect was lost by mutation from D to E (KGE instead of KGD). This suggests that the COL15 domain of collagen type XVII represents a specific collagenous structure, unable to interact with the cellular receptors for other collagens. After being shed from the cell surface it may support keratinocyte spreading and migration.

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YHTEENVETO - Finnish summary

Integriinit reseptoreina fibrillaarisille ja transmembraanisille kollageeneille

Eläinten evoluutiossa tapahtui suuri monimuotoistuminen yksisoluisten (Protozoa) eläinsolujen kehityksen erkaantuessa monisoluisiksi eläimiksi johtavaan kehityshaaraan (Metazoa). Tähän kehitykseen sisältyi myös solunulkoisen sidekudoksen ja tätä sidekudosta sitovien solunpinnan reseptiivisten valkuaisaineiden ilmaantuminen. Yhtenä tärkeimpänä askeleena tässä kehityksessä pidetäänkin kollageenien ja niihin sitoutuvien integriinien syntymistä.

Integriinit ovat joukko transmembraanisia glykoproteiineja, jotka välittävät solujen välisiä ja/tai solujen ja soluväliaineen välisiä vuorovaikutuksia. Integriinit rakentuvat toisiinsa ei-kovalenttisesti sitoutuneesta α- (α1-11, αIIb, αD, αL, αM, αX, αE, aV) ja β-monomeerista (β1-8), jotka voivat muodostaa toiminnaltaan 24 erilaista heterodimeeriä. Nämä rakenteet osallistuvat elimistön normaaliin toimintaan säädellen solujen kasvua, migraatiota, erilaistumista ja fenotyyppiä integroimalla solun sisäisen tukirangan ja signalointiin osallistuvat molekyylit solun ympäristöön. Tämä vuorovaikutus on kaksisuuntaista viestien kulkiessa ympäristöstä solun sisään ja toisaalta solusta ympäristöön integriinien aktiivisuuden säätelyn muodossa. Tunnetaan myös joukko sairaustiloja, kuten kroonisen tulehduksen synty, syövän invasiivisuus ja metastointi, joissa integriinien määrä tai toiminta on muuntunut.

Valtaosan soluväliaineesta muodostaa kollageeni, josta tunnetaan 6 erilaista alatyyppiä ja edelleen lähes 30 erilaista tyyppiä. Alatyyppejä ovat fibrillaariset kollageenit (esim. tyypit I, II, III ja V), verkkomaisia rakenteita muodostavat kollageenit (esim. tyyppi IV), FACIT -kollageenit (fibril-associated collagens with interrupted triple helices, esim. tyyppi IX), filamentteja muodostavat kollageenit (tyyppi VI), ”ankkuroivia fibrillejä muodostavat kollageenit” (tyyppi VII), transmembraaniset kollageenit (esim. tyypit XIII ja XVII) ja multipleksiineiksi kutsutut kollageenit (esim. tyyppi XV). Kollageeneja sitovia integriinejä tunnetaan neljä: α1β1 ja α2β1, sekä ryhmän uusimmat tulokkaat kondrosyyteistä löydetty α10β1 ja sikiökautisista lihassoluista löydetty α11β1. Useimpien kollageenien integriinireseptoreita ei tunneta. Soluväliaineen ligandeina integriineille toimivat kollageenien lisäksi laminiinit, fibro- ja vitronektiini sekä eräät immunoglubuliiniperheeseen kuuluvat solukalvon proteiinit. Sileille lihassoluille on tyypillistä α1β1-integriinien ekspressio, kun taas epiteelisoluille ja verihiutaleille ovat ominaisia α 2β1-integriinit. Useimmat solutyypit, kuten fibroblastit, osteoblastit, kondrosyytit ja lymfosyytit kuitenkin ilmentävät pinnallaan sekä α1β1- että α2β1-integriinejä ja ligaation onkin osoitettu saavan aikaan erilaisia vasteita soluissa.

Osatyössä I luotiin transfektiotekniikalla joko α1β1- tai α2β1-integriinejä ekspressoivat CHO-solulinjat, joilla toteutettiin solutason leviämiskokeet. Rekombinanttitekniikalla tuotettiin integriinidimeerin α 1- ja α 2-alayksikön ensisijaisesti kollageenin tunnistuksesta ja sitomisesta huolehtivat alueet, ns. αI-domeinit, jotka leimattiin europiumilla. Leimattujen proteiinien annettiin kiinnittyä erilaisilla kollageenialustoilla, ja signaali mitattiin aikaerotteisena fluoresenssina. Sitoutumis- ja leviämiskokein tutkittiin integriinien spesifiteettiä

kollageenityypeillä I, II, III, IV, V ja XIII, joita käytettiin monomeereina. CHO-α1β1-solujen todettiin leviävän nopeammin verkkomaisia rakenteita muodostavalla tyvikalvon kollageenilla IV verrattuna fibrillaarisiin kollageeneihin. Vastaavasti α2β1-integriini suosii tyypin I kollageenia, mutta tunnistaa myös tyypin IV kollageenin. Solujen käyttäytyminen näillä kontrolleina toimivilla kollageeneilla oli odotetun mukaista. Sekä tyypin I että tyypin IV kollageeni sisältää α1β1- tai α2β1-integriineille raportoidut $GLP^{(OH)}GER$- ja $GFP^{(OH)}GER$-tunnistussekvenssit. α2β1-integriiniä ekspressoivat solut pystyvät leviämään myös fibrillaarisilla tyypin II, III ja V kollageeneilla, mutta ei transmembraanisella kollageenilla XIII. Tyypin II kollageeni sisältää lajista riippuen joko $GLP^{(OH)}GER$-sekvenssin tai molemmat edellä mainitut tunnistussekvenssit. Huomattavaa on, että tyypin III, V ja XIII kollageenit eivät sisällä tunnettuja tunnistussekvenssejä, viitaten vielä tuntemattoman tunnistusmekanismin olemassaoloon. α1β1-integriinin spesifiteetti on erilainen. Kollageeni II ei indusoi CHO-α1β1-solujen leviämistä lainkaan, ja leviämisen induktioon kollageenilla V tarvitaan suurempi kollageenipitoisuus. Tyypin XIII kollageenin α1β1-integriinit tunnistavat tehokkaasti. Sitoutumiskokeiden tulokset vastaavat pääosin leviämiskokeen löydöksiä. Huomattavaa kuitenkin on, että vaikka α1-alayksikön αI-domeini tunnistaa kollageenityypin II, se ei riitä indusoimaan CHO-α1β1-solujen leviämistä. Tämä viittaa ligandin tunnistuksessa ja sitomisessa integriiniä avustavan reseptorin olemassaoloon.

Osatyössä II tuotettiin rekombinanttitekniikalla integriini α2-alayksikön αI-domeineja, joiden potentiaalisesti ligandin sitomiseen osallistuviin aminohappoihin tehtiin substituutiomutaatioita (D219A, D219N, D219R, D259N, D292N, E299Q) ja αC-heliksin deleetio. Mutatoiduilla proteiineilla tehtiin sekä europium-leimaukseen perustuvia sitoutumiskokeita että reaaliajassa IAsys-biosensorilla todennettavia mittauksia. Leviämiskokeita varten luotiin CHO-solulinja, joka ekpressoi D219N-, D292N- tai D219N/D292N-mutatoidun α2β1-integriinin. Ligandeina tutkimuksissa käytettiin monomeerisia tyypin I ja IV kollageeneja. IAsys-mittauksissa αC-heliksin deleetio muuttaa α2I-domainin sitoutumiskinetiikkaa. Mutantti pystyy sitomaan tyypin I kollageenia ylimäärin verrattuna villityyppiin, mutta sitoutumisen affiniteetti on vain viidennes verrokista. Myös αI-domeinin spesifiteetti muuttuu sitoutumiskokeissa. Viitaten erilaiseen fibrillejä ja verkkomaisia rakenteita muodostavien kollageenien sitoutumismekanismiin αC-heliksmutantti suosii kollageenia IV villityypin suosiessa tyyppiä I. Spesifiteetin muutos muistuttaa α1I-domainin sitoutumista, ja onkin mahdollista, että osa α1I- ja α2I-domeinin eroista määräytyy αC-heliksin perusteella. Negatiivisesti varautuneet aminohapot, jotka ympäröivät Mg^{2+}-sitoutumispaikkaa, osallistuvat tyypin I kollageenin sitomiseen. Lähes kaikki substituutiomutaatiot vähensivät sitoutumista 40-95 % verrattuna villityyppiin. Ainoastaan E256Q-mutaatiolla ei ollut vaikutusta. Leviämiskokeessa tyypin I kollageenillä yksittäisten substituutiomutaatioita ekspressoivien solujen leviämisessä ei ollut merkitsevää eroa verratuna villityypin CHO-α2β1-soluihin. Tuplamutatoitujen solujen leviäminen oli kuitenkin merkittävästi hitaampaa kuin verrokin, viitaten useiden metallin sitoutumispaikkaa ympäröivien aminohappojen osallistumiseen tunnistus- ja sitomistehtävään. Huomattavaa on että, mutaatioilla ei ollut vaikutusta solujen leviämiseen tyypin IV kollageenilla.

Eksosytoottisen erittymisen ja propeptidien katkaisun jälkeen fibrillaaristen kollageenien esiintyminen yksittäisinä monomeereina

soluvälitilassa on lyhytaikaista. Ne muodostavat spontaanisti tiiviitä, tyypillisesti elektronimikroskopiassa juovittaisia säikeitä sekä *in vivo* että *in vitro*. Osatyössä III on ensikertaa vertailtu α1β1- ja α2β1-integriinin sitoutumista monomeerisen tyypin I kollageenin ja fibrilleiksi järjestäytyneen kollageenin sitoutumiseen. Tutkimus toteutettiin pääosin sitoutumis- ja leviämiskokein. Tuloksia täydennettiin elektronimikroskopialla ja kollageenigeeliin perustuvin kontraktiokokein. Tyypin I kollageenin fibrillogeneesi *in vitro* indusoitiin neutraloimalla kollageeni-preparaatti ja lämmittämällä + 37 °C:een, ja säikeiden muodostumista seurattiin absorbanssi-mittauksin. Kollageenin järjestäydyttyä fibrilleiksi sekä α1I- että α2I-domeinien affiniteetti kollageeniin laskee. Ainoastaan α2β1-integriini pystyy sekä leviämään fibrillaarisella kollageenilla että kontraktoimaan fibrillaarista kollageenigeeliä. α1β1-integriini pystyy tunnistamaan tehokkaasti ainoastaan momomeerista tyypin I kollageenia, mikä viittaa sen toimintaan lähinnä kollageenisynteesin negatiivisena palautesäätelijänä.

Tyypin XVII kollageeni esiintyy sekä transmembraanisena että liukoisena muotona soluvälitilassa. Sen ekstrasellulaariosan pisin kollageeninen jakso, COL15-domeini, tuotettiin rekombinanttiproteiinina osatyössä IV. Muista kollageeneista poiketen, sitoutumis- ja leviämiskokeissa todettiin, että α1β1- ja α2β1-integriinit eivät tunnista domeinin natiivia, kollageenista rakennetta. Myös integriini α10β1-integriinin sitoutuminen tarkastettiin. Alhaisen sulamislämmön vuoksi COL15-domein denaturoituu jo fysiologisessa lämpötilassa, mikä saattaa johtaa koko liukoisen osan denaturaatioon. Denaturoituneella domeinilla tehtiin funktionaalisten vasta-aineiden käyttöön perustuvia leviämiskokeita. Rakenteen denaturoiduttua se tunnistetaan α5β1- ja αVβ1-integriinien toimesta. Näitä integriinejä ekspressoivat HaCat keratinosyytit pystyvät leviämään ja migroimaan denaturoidulla COL15-domeinilla, mikä voidaan estää synteettisillä RGD-peptideillä. RGD-tähteiden sijaan jokainen COL15-domeini sisältää yhteensä 12 rakenteellisesti samankaltaista KGD-sekvenssiä. Aktiivisten tähteiden määrittämiseksi suunniteltiin 22 synteettistä peptidiä kattaen koko COL15-domeinin sekvenssin. Kolme KGD-tähdettä sisältävää peptidiä osoittautui inhiboivan HaCat-solujen leviämistä. Tämä tunnistusmekanismi on ainutlaatuinen kollageeniperheessä.

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