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Donald Gullberg *Editor*

I Domain Integrins

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Preface

What Has Been

The integrin family is composed of 24 members [5]. Ten years ago we published a book devoted to the nine αI domain integrin subunits [24]. These are shown in Fig. 1. I am pleased that most of the original authors have been able to contribute to the updated version.

In 2003, the knockout mouse phenotypes for all of the αI domain integrins had not yet been published. They are now. The phenotypes of mouse strain deficient in individual αI integrins are summarized in Table 1.

During the last decade we have learned more about the role of β2 integrins in leukocytes and in leukocyte adhesion deficiencies [29, 27], and the role of β7 integrins in different subsets of immune cells [20]. Much of this knowledge would not have been possible without the use of animal models and have generated results which could not have been predicted from in vitro analyses. Separate from the interesting results in disease models, analyses of αE knockout mice indicate that there is a missing ligand that has not yet been identified for this integrin [20]. Indeed, in human skin and oral mucosa, there is evidence of a ligand for αEβ7 other than E-cadherin [30].

Regarding the role of collagen-binding integrins the knockout phenotypes of mice deficient in integrin α10 and α11, respectively, have now been published [6, 45] and interestingly the enigmatic DDR collagen receptors have recently been shown to affect the function of collagen-binding integrins [1, 53, 62]. In coming years we are likely to learn more about the cross-talk of collagen-binding integrins with other receptor groups. Maybe most surprising in the field of collagen receptors are the relatively mild phenotypes seen in individual knockout strains and the limited role collagen-binding integrins appear to play in classical connective tissue diseases like fibrosis. This is in contrast to the phenotypes observed for different members of the collagen family, where mutants are characterized by major structural defects impacting tissue structure during development and tissue integrity in adult animals [63]. This discrepancy between collagen and collagen receptor-knockout mouse phenotypes is summarized in Table 2. Interestingly, a recent α10 integrin mutation in dogs have indicated that collagen-binding integrins in the muscoskeletal system might have much more severe phenotypes in larger animals/humans compared to the mild integrin phenotypes observed in collagen-binding integrin deficient mice [33].

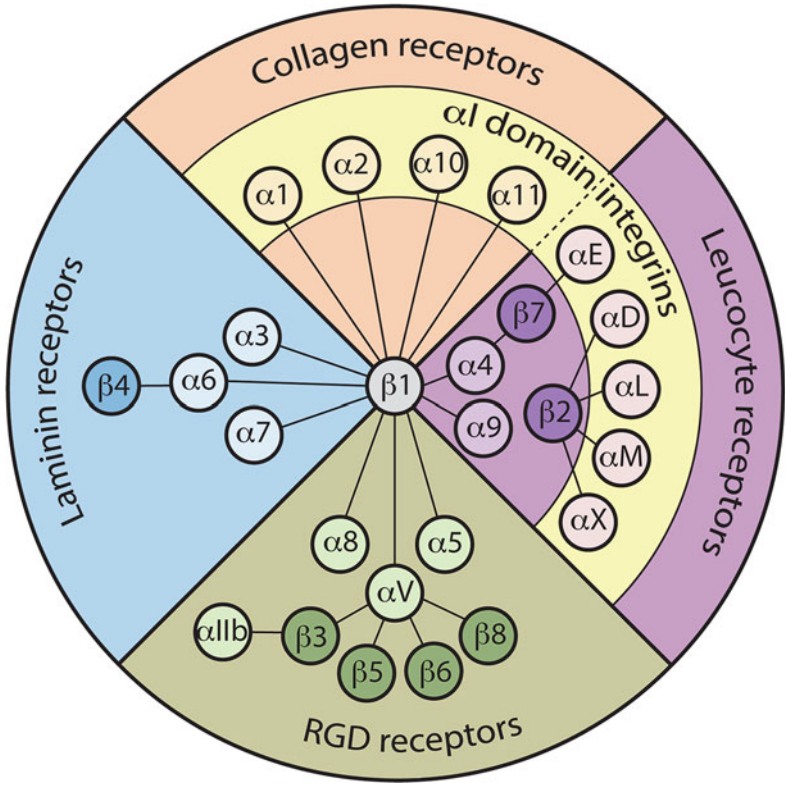

Fig. 1 The integrin family

Table 1 αI integrin knockout phenotypes

Integrin subunit	Distribution	Ligands	Knockout viability	Knockout phenotype
Collagen receptors				
α1	Endothelial cells, smooth muscle cells, fibroblasts, and more cell types [19]	Collagens	+	Normal development [19], hypocellular dermis [18, 47], isolated cells display defect in collagen IV cell attachment
α2	Platelets, epithelial cells, endothelial cells [65], mesenchymal stem cells [44], fibroblasts, and more cell types	Collagens	+	Mild mammary gland phenotype, otherwise normal development [13, 28], cell attachment defect to collagen I of isolated platelets [49], needed for thrombus stabilization [32]
α10	Chondrocytes and subsets of junctional fibroblasts [9, 10]	Collagens	+	Mild cartilage phenotype [6]
α11	Subsets of fibroblasts [46, 55, 57], cancer associated fibroblasts [64], increased levels on myofibroblasts [11], developmental expression in odontoblasts, mesenchymal stem cells [44], induced in cultures of mesenchymally derived cells including myoblasts (do not express α11 in vivo) [25]	Collagens	+	Defective incisor eruption [45], dwarfism [8], increased mortality
Leucocyte receptors				
αD	Macrophages and eosinophils [23, 56]	ICAM-3, VCAM-1	+	Fertile, no gross abnormalities, mild T-cell phenotypic changes [61]
αE	Intraepithelial lymphocytes, some circulating lymphocytes, lamina propria lymphocytes, subsets of CD4+ T-cells, CD8+ T-cells, dendritic cells, mast cells [12, 31, 35]	E-cadherin, uncharacterized ligand	+	Impaired development of gut associated lymphoid tissue [51]
αL	All leucocytes [54]	ICAM-1,-2,-3,-4,-5, JAM-1	+	Splenomegaly and reduced lymph node size [50], increased white blood cells counts [15], reduced lymphocyte homing [7], reduced neutrophil adhesion [15], Treg and NKT cell development affected [42, 59] reduced T-cell proliferation and co-stimulation [21, 50, 52]
αM	Monocytes, macrophages, NK cells, neutrophils, and subsets of T-cells [22, 34, 41]	iC3b, fibrinogen, and more ligands	+	Neutrophil phagocytosis and degranulation reduced [14, 40], impaired mast cell development and function [48], excessive macrophage and dendritic cell toll-like receptor signaling [26, 4], excessive Th17 differentiation [17]
αX	Monocytes, macrophages, dendritic cells, NK cells [41]	iC3b, fibrinogen and more ligands	+	Fertile, no gross abnormalities, affects monocyte firm adhesion [60]

Table 2 Phenotypes of mice deficient in fibrillar collagens and integrin collagen receptors

Ligand			Receptor		
Fibrillar collagen	KO phenotype in mouse	KO phenotype in human	Putative collagen receptor in vivo	Correlation KO phenotypes collagen/ receptor in mouse	KO phenotype in human/dog
I	Mov13 mice [39]: embryonic lethality E12-14, major blood vessel rupture	EDS[a] VIIA, EDS VIIB, OI[b], osteoporosis, joint hypermobility	$\alpha2\beta1$ $\alpha11\beta1$	Not in single integrin mutant strains	?
II	Perinatal lethality [2, 36] short long bones, rudimentary vertebral arches, lack of inter-vertebral discs, notochord defect	Lethal achondrogenesis II, osteochondrodysplasia, osteoarthrosis	$\alpha1\beta1$ $\alpha2\beta1$ $\alpha10\beta1$	$\alpha10$ integrin mutation [6], mild cartilage defect $\beta1$ integrin [3], severe cartilage defect	Chondrodysplasia in dogs, integrin $\alpha10$ mutation [33], severe cartilage phenotype
III	Neonatal lethality [38], 5 % survival with shorter lifespan, intestinal defect, skin lesions, arterial rupture	EDS IV, arterial aneurysms	$\alpha2\beta1$ $\alpha11\beta1$	?	?
V	Embryonic lethality E10-11 [58], cardiovascular insufficiency, lack of collagen fibrillogenesis	EDS I, EDS II	$\alpha2\beta1$ $\alpha11\beta1$	?	?
XI	Cho mice: perinatal lethality by asphyxia [37], weak tracheal cartilage, short snout and mandible, cleft palate, short limbs, externally rotated distal portion of hindlimbs	Schmid chondrodysplasia, non-syndromic hearing loss, osteoarthrosis	$\alpha2\beta1$ $\alpha10\beta1$ $\alpha11\beta1$	$\alpha10$ integrin mutation [6], mild cartilage defect $\beta1$ integrin [3], severe cartilage defect	Chondrodysplasia in dogs, integrin $\alpha10$ mutation [33], severe cartilage phenotype
XXIV	?	?		?	?
XXVII	Mutant transgene [43]: perinatal lethality, lung defect, chondrodysplasia	?	$\alpha2\beta1$ $\alpha11\beta1$	?	?

a Ehlers–Danlos syndrome
b Osteogenesis imperfecta

What Will Come

As in all biological fields, techniques are moving the fields forward as methods become more refined. We now have access to new tools, enabling studies at the nano-scale, and reagents designed to block integrin function can thus be applied to nanoparticles.

In the cancer field, the microenvironment is taking center stage, and here integrins on fibroblasts are predicted to play important roles in paracrine signaling, in regulating tissue stiffness [16] and matrix remodeling.

With exome sequencing of rare genetic diseases becoming more widely used, this will enable new human integrin mutations to be tested in disease models. The development of new molecular techniques to more easily generate mutations in vivo might also contribute to more animal disease models being established.

New technologies, new mouse models in combination with analyses of αI integrins in larger animals/humans are thus predicted to increase our knowledge about this group of receptors. With these things in mind we look forward to another 10 years of research with αI domain integrins.

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Bergen, March 2014 Donald Gullberg

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Contents

Evolution of Integrin I Domains

1

Mark S. Johnson and Bhanupratap Singh Chouhan

Abstract

In humans, an ~200-residue "inserted" I domain, a von Willebrand factor A domain (vWFA), buds out from the β-propeller domain in 9 of 18 integrin α subunits. The vWFA domain is not unique to the α subunit as it is an integral part of *all* integrin β subunits and many other proteins. The βI domain has always been a component of integrins but the αI domain makes its appearance relatively late, in early chordates, since it is found in tunicates and later diverging species. The tunicate αI domains are distinct from the human collagen and leukocyte recognizing integrin α subunits, but fragments of integrins from agnathastomes suggest that the human-type αI domains arose in an ancestor of the very first vertebrate species. The rise of integrins with αI domains parallels the enormous changes in body plan and systemic development of the chordate line that began some 550 million or more years ago.

Keywords

Integrin · I-domain · Evolution

1.1 Introduction

Integrins are cell-surface receptors that straddle the plasma membrane by means of a single transmembrane helix in each of two subunits. Integrins in general function to mediate cell-cell and cell-matrix interactions (for a review, see Eble and Kühn [26]); furthermore, integrins are mechanical receptors. Thus, they respond to both external and internal ligands through large changes in receptor conformation that are tightly coupled to function. Integrin signalling is also bi-directional [36], meaning that the presence or absence of molecular interactions in either the cytoplasm or in the extracellular space can modulate the integrin-mediated functions within the other compartment. Thus, integrins are dynamic communicators of both the intracellular wishes of a cell for its extracellular environment as well as mediating environment feeding back to intracellular signalling and downstream intracellular events.

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Integrins are $\alpha\beta$ heterodimers and in humans there are 18 α subunits and 8 β subunits that associate to form 24 non-covalently linked heterodimers. The human α subunits range between about 1,050 and 1,190 residues and β subunits between about 770 and 800 residues (with the exception of β4 at over 1,800 amino acids, having a unique additional C-terminal domain). Integrin α and β subunits are known to mutually form a large, N-terminal, extracellular multi-domain "ectodomain" structure, with a globular head and tails, followed by a transmembrane region and relative short C-terminal cytoplasmic sequences. This view was initially based on both electron microscopy data [12, 62] and the analysis of sequences of integrin subunits and the location of a single hydrophobic stretch of residues towards the C-termini of each subunit identified as the presumptive membrane-spanning helical region (e.g. [4, 62]). The complex domain structure [5, 54, 102]; see e.g. Fig. 9.1 in [43] of each subunit appears to be key to the overall dynamic structural changes [55, 58, 103] that are associated with integrin functions and the ability to communicate signals from inside-out and from outside-in.

From the earliest X-ray studies, i.e. αL [72] and αM [52, 51], focus was placed on the human αI domains. Today, structures are also known for I domains of integrin α2 and α1 without (e.g. [27, 76, 64]) and with bound collagen-like triple helical peptides [14, 28], and αX [95]; the latter also within the context of the αXβ2 ectodomain [100]. A region near the N-terminus of the β subunit was predicted to be a von Willebrand Factor type domain based upon the analysis of sequence data [52, 90, 92]. The overall structure of the integrin subunits has thus been detailed by multiple three-dimensional structures of ectodomains and other parts thereof (transmembrane and C-terminal regions) as revealed using structural techniques including X-ray crystallography, NMR spectroscopy, and cryoelectron microscopy. Because the integrins undergo dynamic structural changes, the available structural snapshots provide only a partial description of their full range of functional conformations.

The X-ray structure of the ectodomain of human integrin αVβ3 (PDB code: 1JV2; [102]) was the first reported structure of the extracellular regions of the α and β subunits and their mutual interactions, and those features have been found to be generic features of integrins also observed in the subsequent structures solved for, e.g. the ectodomains of αIIbβ3 [99] and αXβ1 [100] and the N-terminal headpieces (includes the β propeller of the α subunit and β I domain of the β subunit) of α4β7 [105] and α5β1 [60]. Ligand complexes [82, 104] with peptides, having e.g. the "RGD" recognition sequence, pinpointed the narrow binding region suitable for loop recognition, located between the β propeller of the α subunit and the βI domain of the β subunit.

The ectodomain structure of αXβ2 [100] illustrates the relative disposition of the αI domain and revealed the high exposure of the binding site of the αX I domain that would allow integrins to recognize an entirely new class of ligands, such as immunoglobulin fold domains and triple-helical collagens. The αI domain buds out of the N-terminal ~440-residue 7-bladed repeat β-propeller domain within the loop between blades 2 and 3. The β propeller is highly conserved and has been identified in some non-integrin bacterial sequences [15, 40].

In humans, I domains are present in one-half of the integrin α subunits. Four of these nine α subunits, namely α1, α2, α10, and α11, partner with the β1 subunit and are generally referred to as collagen receptors. Five other α subunits, αD, αE, αL, αM and αX, are associated with cells of the immune system, where αE forms heterodimers with β7 but αD, αE, αM, and αL form dimers with the β2 subunit.

The integrins and their evolution were already key topics of study from around the mid-1980's as functional, sequence and structural studies were taking place in multiple laboratories, and the "inserted" I domain [50] or "A" domain [4] was recognized as a novel addition in multiple integrin α subunits. In 2003 (Johnson and Tuckwell), based on the fairly high sequence similarity seen between orthologous human and bony fish integrin subunits with αI domains and the presence of a single αI domain detected in tunicates [57], it was fully expected to find broad coverage of αI domains and even human orthologues throughout the bony fish,

sharks and rays, lamprey and hagfish, and even within the invertebrate chordates. Over more recent years, considerable genomic data has become available and in 2014 we have the benefits of all of the accumulated data and their interpretation from many sources, leading to a more coherent view of integrin and especially αI domain evolution. These data not only clarify the likely range of αI domains within extant species, they also help clarify the development of α subunits with I domains orthologous to the types seen in humans and other higher vertebrates.

1.2 Relationships Among Integrin α and β Subunits

In order to set the stage for the appearance and diversification of α subunits containing I domains it is important to recount the evolutionary range of integrin α and β subunits. Complete α and β subunits are present in the earliest metazoans (reviewed in [43]), which suggested that integrins would even predate the first animals. Integrin-like domains and folds-types corresponding to individual integrin domains are also found in some prokaryotes. For example, compared with the integrins the repeats within the β-propeller domain of prokaryotes are even more similar to each other in terms of the loops and calcium binding sites; the latter present in all 7 repeats instead of only 3 or 4 repeats seen with the integrins [15]. But, the domain is present in only some prokaryotes and there is the possibility that they were acquired through non-Darwinian means, i.e. horizontal gene transfer. Moreover, where it was detected the β-propeller domain was present in protein sequences whose function is unknown and without the hallmarks of any other integrin subunits. Similarly, most other domains from which integrins are composed of (e.g. vWFA domain—in both α and β subunits, epidermal growth factor domains—in the β subunit, and immunoglobulin domains—in both α and β subunits) can be located within prokaryotic proteins (reviewed in [43]).

The earliest integrin subunits have been reported in single-cell eukaryotes, the choanozoa, which are the closest relatives of the animals. An integrin-like β subunit fragment was identified in *Ministeria vibrans* [80] and *Capsaspora owczarzaki* contains four α and β integrin subunits each [79, 86], and these sequences can be identified through a simple BLAST search even though, for example, a β subunit shares only between 11 and 21 % sequence identity with a set of known integrin β subunits [43].

Integrin subunits occur across the full range of invertebrates [10] and integrin α and β subunits have long been known to exist within the earliest-appearing animals, e.g. sponges, corals and jellyfish [9, 59, 67, 73]. Similarly, integrin α and β subunits have been identified in the genomes of other early diverging metazoans, including other sponges *Oscarella carmela* (Porifera [63]) and *Amphimedon queenslandica* [84], the placozoan Trichoplax adhaerens [78, 83], the coral *Acropora millepora* (Cnidaria [48] and the sea anemone *Nematostella vectensis* (Cnidaria [48]). Like *C. owczarzaki*, these early metazoans have multiple copies of subunits: For example, *T. adhaerens* has two α subunits and one β subunit; and *N. vectensis* has three or more α and β subunits.

Based on sequence data, evolutionary relationships including multiple phylogenetic representations of the relationships among integrins subunits have been described over the past 25 years [20, 32, 10, 30, 33, 37, 29, 34, 42–43, 87] among others), and they are in close agreement with each other although the naming of individual sequences may have changed and clustered groups may differ somewhat. Hynes and Zhao [37] and Hughes [33] segregated the α subunits with respect to the *Drosophila melanogaster* α subunits into the laminin receptor-like "PS1", the RGD-recognising "PS2" clades and a unique set of duplicated α subunits in the "PS3" clade, containing only invertebrate members (see Bökel and Brown [11] for a review). "Position Specific" antigens from *D. melanogaster* had been known for some time before the full complement of integrin α subunits was defined by the fruit fly

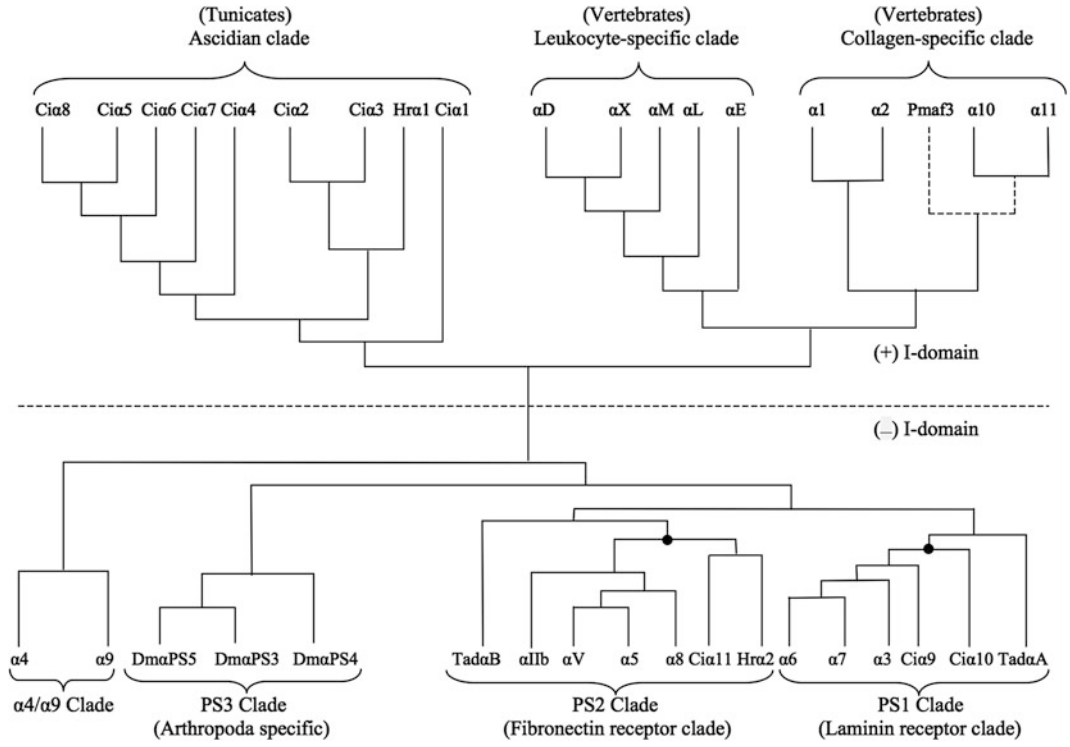

Fig. 1.1 Summary tree for integrin α subunits displaying the phylogenetic relationship among sequences from human, the sea lamprey *P. marinus* (Pmaf3), the tunicates *C. intestinalis* (Ci) and *H. roretzi* (Hr) as well as the arthropod *D. melanogaster* (Dm) and the placozoan *T. adhaerens* (Tad). This summary tree is based on a sequence comparison of 38 vertebrate and invertebrate sequences. The I-domain clade consists of nine representative sequences each from human and the tunicates. The branching patterns for the two *T. adhaerens* sequences have low bootstrap support. The branch lengths in the tree are arbitrary

genomic sequence [1, 75]. The summary tree for integrin α subunits represented in Fig. 1.1 reflects this classification scheme. The two α subunits of the nematode *Caenorhabditis elegans*, Ina-1 and Pat-2, respectively cluster with the PS1 and PS2 clades. In human, α3, α6 and α7 are found in the PS1 group and αIIb, αV, α5 and α8 cluster within the PS2 clade. The αA and αB subunits of the placozoan *T. adhaerens*, depending on the other sequences being compared, cluster respectively with the PS1 group and PS2 group (Fig. 1.1), or together with the PS1 clade [43], but the bootstrap significance in either case is low. In addition to the human members of the PS1 and PS2 clades, there is an α4/α9 clade and clades involving α subunits with inserted I domains (Fig. 1.1).

The β subunits have been clustered into two vertebrate clades, vertebrate A and vertebrate B, as well as an invertebrate clade [10, 34]. Of the human β subunits, β1, β2 and β7 belong to the vertebrate A clade and β3–β6 and β8 belong to the vertebrate B cluster. The β subunits of the vertebrate A clade pair with α subunits having the inserted I domain, but in human β1 is overall the most promiscuous subunit, pairing with the four collagen receptor α subunits plus eight α subunits lacking an I domain. Human β7 clusters with both αE and α4 (which also pairs with β1), the latter lacking the I domain; and human β2 partners only with the immune cell subunits αD, αL, αM and αX. The third major cluster contains invertebrate β subunits, e.g. the two β subunits, βv and βvPS from *D. melanogaster* and pat-3 from *C. elegans* (see Fig. 1.4, [34]). The invertebrate cluster also includes 4 of 5 β subunits in the genome [21] of an early diverging invertebrate chordate—the tunicate *Ciona intestinalis* (Urochordate). A fifth β subunit—Ciβ5—clusters within the vertebrate B

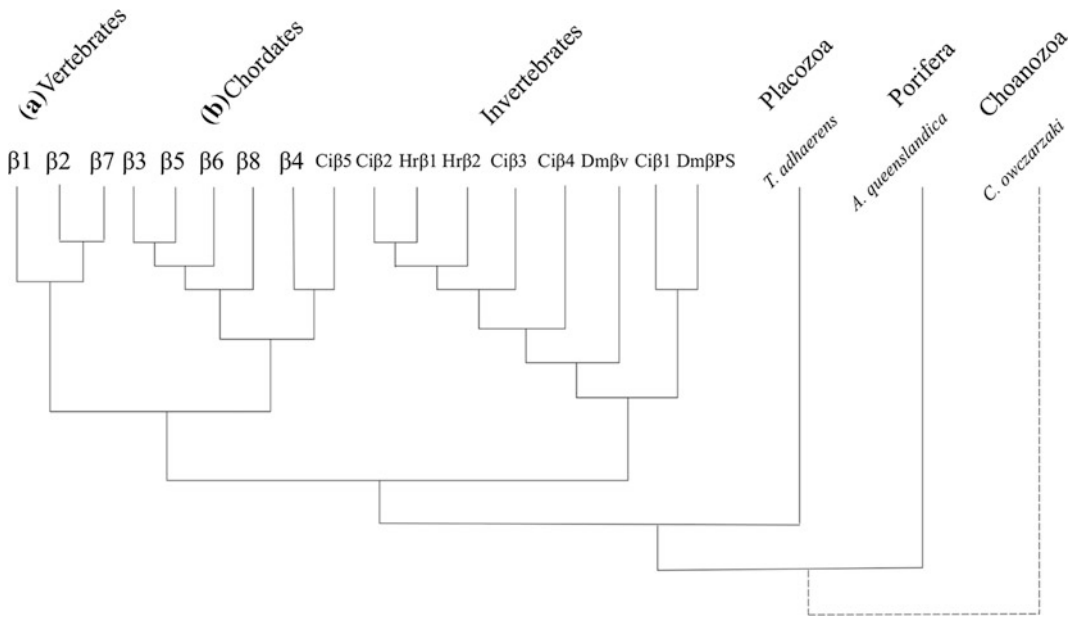

Fig. 1.2 Summary tree for integrin β-subunits displaying the phylogenetic relationship based on 40 vertebrate and invertebrate sequences including sequences from the poriferan *A. queenslandica* and the choanozoan *C. owczarzaki*. The integrin β-subunit sequences derived from *C. owczarzaki* have been utilized to root the tree. The branch lengths in the tree are not to scale

clade [34], so the vertebrate B clade extends at least to one invertebrate species; we have thus labeled it the "Chordate" group to reflect Ciona's inclusion (Fig. 1.2). Representatives of the earliest diverging metazoans, *T. adhaerens* and *A. queenslandica* cluster as outliers of all of the other species when the single-cell eukaryote *C. owczarzaki* is used as an out-group to root the tree (Fig. 1.2).

1.3 Human Integrin α Subunits with the I Domain Have Unique and Common Features

The clustering of the nine human α subunits containing the I domain (Fig. 1.1) follows the phylogeny described by Hughes [33]. When the sequences of full-length integrin α subunits are compared two major clusters are observed, with the collagen-binding cluster ((α1, α2) (α10, α11)), separating cleanly from the integrin α subunits of the immune system: ((((αD, αX)αM)αL)αE).

In contrast to the human integrins, no I domains were found in the α subunits from the genomes of either *C. elegans* or *D. melanogaster* [37], nor in sequences from other invertebrates including very early diverging metazoan species like the placozoa, porifera and cnidarians. Neither are αI domains found among the first diverging deuterostomes—the echinoderms—that directly precede the appearance of the chordate line. Orthologues of the human αI-containing subunits (and other subunits) were, however, identified in sequences from mouse, other mammals, birds and amphibians [33, 42, 96], among others) and likely orthologues in bony fish were identified [42].

One characteristic feature of integrin αI and βI domains (and of many vWFA domains) is the metal ion dependent adhesion site "MIDAS" (Fig. 1.3) where a metal cation, e.g. Mg^{2+}, is bound. Thus, MIDAS is present in all integrins, but the binding modes and the ligand features that can be recognized in the presence or absence of an αI domain are different [88]. The metal ion at MIDAS is bound by conserved

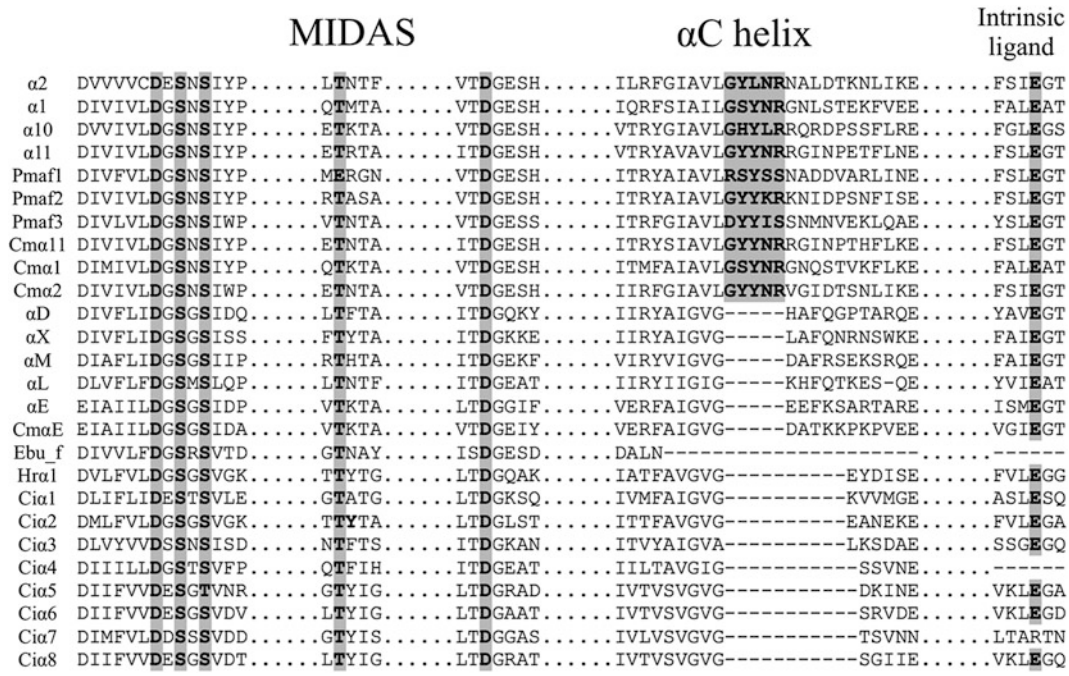

Fig. 1.3 Sequence alignment over regions of representative integrin αI domains with key amino acids highlighted: residues of MIDAS involved in binding the metal cation where ligand binding takes place via a glutamate residue as in the X-ray structures of the α2 I domain with bound $GFOGER_3$ collagen-like triple helical peptide (PDB code: 1DZI; [28] and the αL I domain with bound ICAM3 (PDB code: 1T0P; [81]; the diagnostic region referred to as the αC helix, is formed in the ligand-free form of the α2 I domain (PDB code: 1AOX; [27], but the corresponding region is missing from members of the leukocyte αI clade and the ascidian αI domains. The intrinsic ligand, e.g. E^{336} in the α2 I domain, is located at the opposite end of the domain from MIDAS and binds to MIDAS of the βI domain in the β subunit, forming part of the activation mechanism when external ligands bind to MIDAS at the αI domain of the α subunit

residues of the I domain; in the α2 I domain [27, 28] by ^{151}DxSxS^{155}, T^{221} (and D^{151}) via a water molecule, and D^{254} (Fig. 1.4a). The other coordination positions of the metal ion bind water molecules and the negatively-charged residue from the ligand recognized by the I domain displaces a water molecule and binds directly to the metal ion. The βI domain, in addition to MIDAS, contains the synergistic metal ion-dependent site (SyMBS) and the adjacent to metal ion-dependent adhesion site (ADMIDAS), both of which bind calcium.

In integrins *without* αI domains, the βI domain binds a negatively-charged aspartic acid from the ligand via a positively charged metal ion at MIDAS as seen in the X-ray structures of αVβ3 (cyclic "RGD" peptide, PDB code 1L5G, [104] and αIIbβ3 (fibrinogen γ chain "AGD" peptides: e.g. PDB code 2VDO, and chimeric "RDG" peptide in 2VDR; [82]. In integrins *with* αI domains, an "intrinsic ligand"—a glutamate residue—is conserved across the αI domains (e.g. E^{336} in the α2 I domain; Figs. 1.3, 1.4b) and when ligands bind to MIDAS of αI the glutamate is proposed to bind to MIDAS of the βI domain as a part of the integrin conformational regulatory mechanism [2]; Yang et al. 2004; [44, 100].

In comparison with extrinsic ligand binding to the βI domain, ligands that bind to MIDAS of the integrin αI domain [57] do so through a slightly larger, negatively charged glutamic acid side chain, as illustrated for the two subsets of αI domains in human: The collagen-type, e.g. from the collagen-like triple helical-peptide GFOGER

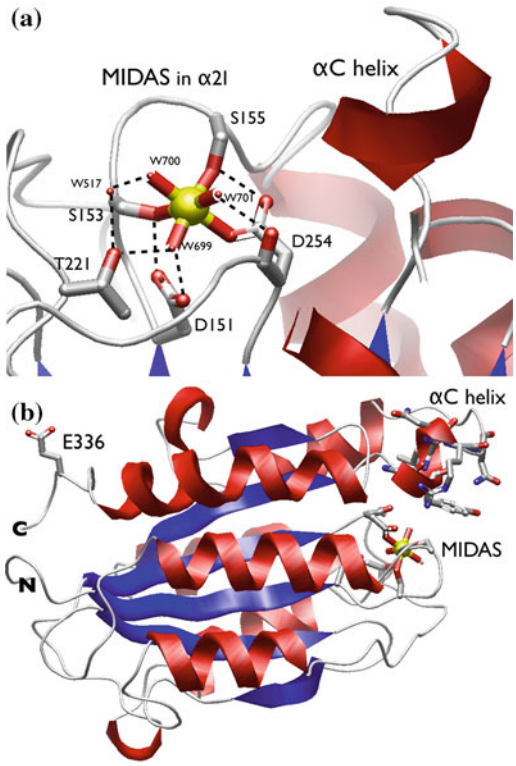

Fig. 1.4 Key features of the αI domain based on the human α2 I domain structure (PDB: 1AOX [27]). **a** The MIDAS site and location of αC helix, including Mg^{2+} (*yellow sphere*), water molecules (*W*) key residues involved in binding directly to Mg^{2+} or via water; dash lines represent likely hydrogen bonds. **b** Relative positions of MIDAS, the αC helix and E336 with respect to the C-terminal (*c*) and N-terminal (*N*) ends of the αI domain, which buds out from the *β* propeller domain of the α subunit. The residues of the αC helix in the α2 I domain are GYLNR, and are shown in ball-and-stick figures. Helices are in *red* and strands in *blue*. The figure was rendered in Bodil [53]

in complex with the α2 I domain (PDB code: 1DZI; [28] and the immune cell recognizing integrin α subunits, e.g. from the immunoglobulin domain of ICAM3 bound to the αL I domain (PDB code: 1T0P; [81]. Note that some "unintended" ligands can bind αI domains in a metal-independent fashion, such as lovastatin to the αL I domain [45], and the "RKKH" motif of a peptide from a snake venom metalloproteinase [38, 68] and echovirus 1 [6, 44, 47, 101] to the human α2 I domain.

Whereas, the MIDAS site is a universal feature of integrin αI domains (Fig. 1.3), and even of the *β*I domain of the *β* subunit and present in some but not all vWFA domains, the collagen-binding αI domains are easily distinguished from those of the immune system and from other vWFA domains by simply examining one key feature of their sequence alignment: Neither the leucocyte-specific αI domains (Fig. 1.3) nor other vWFA domains contain the αC helix present in all of the collagen receptor members. The α2 I-domain structure described by Emsley et al. [27]; PDB code: 1AOX) pinpointed this major difference with the αL and αM I-domain structures—there is an additional helix, αC (Fig. 1.4), in the vicinity of MIDAS whose conformation, along with adjoining regions, changes in response to collagen binding as seen in the α2 I domain bound to a collagen-like triple-helical GFOGER peptide (PDB code: 1DZI; [28]). In the α2 I domain the αC helix corresponds to the sequence ^{284}GYLNR^{288} (GSYNR in α1, GHYLR in α10 and GYYNR in α11). The presence of the αC-helix region is diagnostic of the collagen-binding αI domains, and it is neither found in I domains of the immune system integrins (Fig. 1.3) nor in other vWFA domains and it has been essential for identifying collagen-type integrin αI domains in sequence fragments e.g. from lamprey [16].

1.4 Tunicates and Bony Fish Set Boundaries for Understanding αI Domain Evolution

The answers to two questions posed earlier [31, 42, 40] on the evolution of integrins with αI domains are increasingly being clarified, largely as a result of the genome sequencing studies occurring within chordate species. Firstly, what are the earliest diverging species with integrins having αI domains? And, related to this question—are they orthologues of the human integrins? And, if not, when did the first orthologues arise?

In 2001, Miyazawa et al. identified two integrin α subunits in an early diverging chordate, the ascidian *Halocynthia roretzi* (Urochordate; tunicate). One, αHr2, belongs to the PS2 clade along with the human subunits αIIb, αV, α5, and α8 (see, e.g. [34]), but the second, αHr1, contained an αI domain (Fig. 1.1). This was the first invertebrate I domain to be identified and αHr1 was experimentally associated with the recognition of complement factors in the ascidian immune system [57]. An early phylogenetic reconstruction [42] placed αHr1 as an outlier to both the vertebrate collagen and immune systems clades when the tree was rooted by the position of the non-integrin vWFA3 domain. Furthermore, αHr1 does not have a sequence corresponding to an αC helix (Fig. 1.3).

Soon thereafter, the genome sequence from another ascidian, *C. intestinalis* [21], led to identification of 11 α subunits. Three α subunits lacked the αI domain (Ciα9 and Ciα10 cluster with the PS1 clade and Ciα11 cluster with PS2; Fig. 1.1), but eight others, Ciα1-Ciα8, cluster with αHr1 and separately from both the human leukocyte clade and the collagen receptor clade [29, 34]. The ascidian sequences, individual αI domains or full-length integrins, with the exception of Ciα1 cluster consistently into two main groups using multiple methods (e.g. Bayesian, maximum likelihood, neighbor joining) for phylogeny reconstruction [17], with αHr1 and Ciα2-3 in one group and Ciα6-8 in the other (Fig. 1.1). Based on the phylogenetic reconstructions and closest sequence matches, it is clear that the ascidian integrins are not orthologues of any of the bony vertebrate α subunits having I domains [34, 77]. It was therefore not a surprise that, like αHr1 of *H. roretzi*, none of the *C. intestinalis* sequences have the αC helix characteristic of the collagen-binding αI clade (Fig. 1.3).

Thus, the ascidian data show that some early invertebrate chordates already had integrins with I domains when they diverged within the chordate lineage, but they represent paralogues of the nine human integrins; no orthologous pairs exist. In contrast, at the other end of the spectrum, orthologues of human I domains could be traced back through other mammals, birds, reptiles, amphibians, and even to bony fish [34, 42], suggesting that orthologues of the human integrins with αI domains might be found across the whole range of vertebrate species [42]. Individual integrin sequences from bony fish (e.g. from *Cyprinus carpio*, carp; *Danio rereo*, zebrafish) had also become available as well as genome sequences from e.g. the pufferfish *Takifugu rubripes* [3] and *Tetraodon nigroviridis* [39]. Thus, human integrin subunit orthologues were identified in fish, including α subunits with I domains, and clustered these sequences to the collagen and leukocyte clades (e.g. see [34]). Fish, thought to have undergone an extra round of whole genome duplication in comparison to later diverging vertebrates (see e.g. [22, 89]), also exhibited duplicate isoforms orthologous to the human subunits (e.g. [34]), and today there are orthologous representatives identified in bony fish for nearly all of the human integrin chains and duplicate isoforms are the rule rather than an exception [17].

Thus, the ascidians and the bony fish now provide key and well-established demarcations for integrin αI domain evolution. Urochordates, thought at the time to be the earliest diverging species of the chordate line, have non-human-like integrin subunits with αI domains, whereas human orthologues are present in bony fish. Only three extant groups of organisms were considered to have diverged after the urochordates and prior to the bony fish: The lancelets, the cyclostomes (or agnaths; jawless vertebrates) and the cartilaginous fish. The lancelets, according to established taxonomy, were positioned as the closest living relatives of the vertebrates but this notion has now been challenged by the molecular data.

1.5 Lancelets, the Jawless Vertebrates, the Cartilaginous Fish and the Origin of Vertebrate Orthologues

The last common ancestor of the echinoderms and chordates is estimated to have occurred around 520–550 million years ago according to

the fossil record, and considerably earlier when based on sequence comparisons and phylogenetic reconstructions (see e.g. [8, 24]). There are two extant invertebrate chordate groups, the cephalochordates and the urochordates. The presence of an I-domain containing the α subunit in the urochordate *H. roretzi* supported the idea that α subunits containing I-domains would be found in the "later-diverging" cephalochordates (lancelets), and especially within the agnathastomes (cyclostomes; hagfish and lamprey) and chondrichthyes (sharks and rays) [42, 40]. In 2008, the genome of the lancelet *Branchiostoma floridae* (Cephalochordate; amphioxus; [71]) was reported. A search of the genome revealed integrin α and β subunits but α subunits with I domains were not identified [31].

This then led to the following quandary: If the urochordates have αI domains, then how is it possible that they are absent in the later-diverging lancelet? Interestingly, a controversy on the relative divergence times of the two earliest representatives of the chordates was also underway. The lancelets, based on e.g. morphological features (for a considered review of the non-sequence based evidence, see [85], were long thought to have diverged *after* the tunicates—i.e. after the divergence of *H. roretzi* and *C. intestinalis* and other urochordates from the chordate line. In such a case, it would be difficult to reconcile how the lancelet would have "lost" the integrin subunits with αI domains. However, the comparison of 146 genes across 49 species [69], 1,029 concatenated sequences among the deuterostomes [71], and ∼40 Mb of expressed sequence tags across 21 phyla [25] all concluded that the urochordates, not the cephalochordates, are the closest extant relatives of the vertebrates. Earlier, a similar controversy led to the reassignment of hagfish and lamprey to a monophyletic group based on the molecular data (see, e.g. [65]) and in contrast to the morphological arguments. Considered in this light, the absence of αI domains in echinoderms and all earlier animals, as well as the lancelet, coupled with the αI domain presence in the ascidians, is congruent and pinpoints the origin of the integrin αI domain integration event to have occurred after the divergence of the cephalochordates and prior to the divergence of the urochordates.

Since the urochordates have integrin αI domains that are not orthologues of the human types, the remaining two groups of extant species diverging prior to the bony fish—namely, the hagfish/lampreys (Agnatha; jawless vertebrates) and the sharks/skates/rays/chimera (Chondrichthyes; cartilaginous fish)—should provide evidence for the origin of the human-type integrin αI domains.

Fragments of sequences have appeared from the genomic sequencing of Petromyzon marinus (sea lamprey) and searches against the ENSEMBL data (http://www.ensembl.org/Petromyzon_marinus/Info/Index) yielded several fragments and a near full-length integrin sequence [16]. Phylogenetic reconstructions with the nearly full-length lamprey integrin and the fragments clearly showed that they are not part of the urochordate cluster, nor are they part of the immune cell recognizing set of αI domains; however, they have the αC-helix (Fig. 1.3; [16]) and they do cluster within the collagen recognizing integrin αI domain set that includes human α1, α2, α10 and α11 (Pmaf3 in Fig. 1.1; Fig. 1.5; [17]). Furthermore, preliminary binding studies on expressed sequences suggest that the lamprey sequences bind different collagens and, unlike the α1I domain of *C. intestinalis* [93], the binding is metal-dependent [17]. Thus, the *P. marinus* sequences are functional members of the collagen recognizing set of integrin αI domains; e.g. Pmaf3 branches as an outlier to both the α10 and α11 I domains (Fig. 1.1) and Pmaf1 and Pmaf2 associate with the collagen recognizing αI domains too (Fig. 1.5). A short sequence fragment, an Expression Sequence Tag from *Eptatretus burgeri*—the inshore hagfish, was also identified in searches and may correspond to the N-terminal portion of an αI domain but the sequence ends just at the junction where the αC helix would have begun if present (Fig. 1.3; [16]. Nonetheless, if that fragment does in fact correspond to part of an αI domain then the sequence is clearly not of the urochordate type, nor is it a member of the collagen-binding clade. Instead, it seems most similar in sequence to the

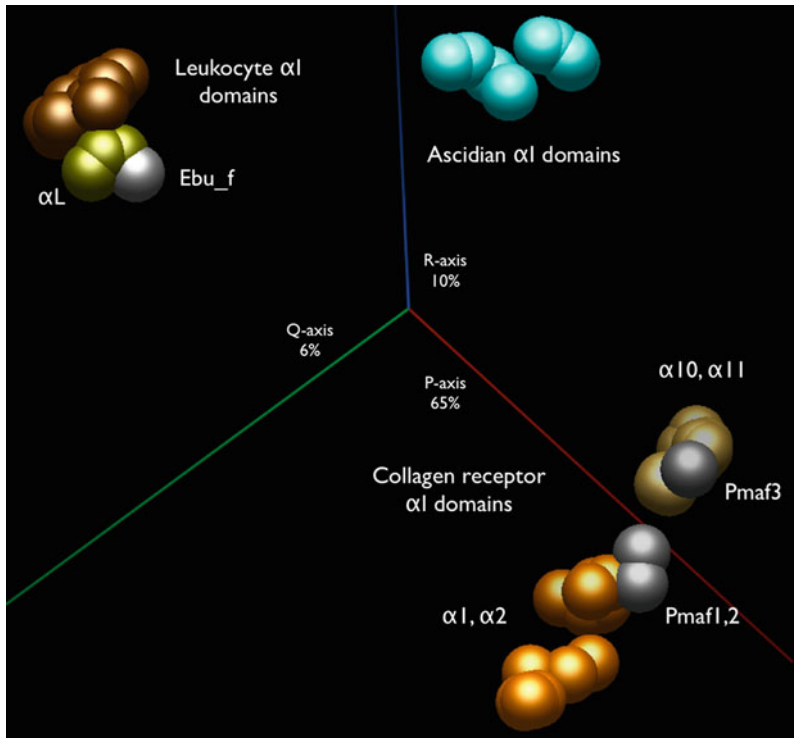

Fig. 1.5 Multivariate analysis showing the mutual relationships among αI domains from human integrins and other vertebrate integrins, ascidians, and fragmentary sequences from lamprey (Pmaf1-3) and hagfish (Ebu_f). Spheres represent individual αI domains and some domains are superimposed at the same location or "behind" other I domains and thus may not be separably visible. Sequences were aligned with Malign [41] and the C program PCA (MS Johnson) was used to compute the three-dimensional projections maximizing the view of the overall variance among the data, generating pseudo-PDB coordinates, displayed and rendered in Bodil [53]. The percentage of the total variance displayed along the P, Q and R axes is shown

leukocyte-binding, human-type integrin αL I domains (Fig. 1.5). Two other C-terminal αI domain fragments were found in searches of sequence data from the genome sequencing of *Callorhinchus milii* (Chondrichthyes; ghost shark, elephant shark). The fragments began at the αC helix junction, having the sequences GSYNR (an α1 orthologue) and GYYNR (an α11 orthologue) of the αC helix, and they correspond closely to human collagen-binding αI domains. Indeed, they represent true orthologues (see Fig. 1.3 for the comparison with the extracts from the full-length sequences) and with the recent published genome data for *C. milii* [94], a large hole in the vertebrate data coverage is plugged; thus, full-length orthologues to the human sequences αE, α1, α2 and α11 are at least present in this cartilaginous fish [17].

1.6 A Nebulous Origin of the Integrin I Domain

The vWFA domain, present as the αI domain is some α subunits and as βI domains in all β subunits, is found in a wide range of other proteins with diverse function (e.g. collagens, complement factors, copines, matrilins, ion channels, protease inhibitors, among others), which are distributed across all of the domains of life [42, 70, 97]. vWFA domains are highly represented in proteins that have roles especially

related to processes within the immune and circulatory systems, and are associated with cell-cell and cell-extracellular-matrix (ECM) recognition, being component domains of adhesion molecules at cell membranes as well as proteins of the ECM (see e.g. [18, 19, 97]), among others.

Despite the wealth of information that exists on vWFA domains, it has so far not been possible to establish the likely source for the vWFA domains inserted into the integrin subunits [42, 91]. The branch orders in trees constructed from sequence comparisons of vWFA domains may be robust for similar members within a clade (according to bootstrapping of the sequence alignments), but the relative branching orders among clades are not reliable [42, 91]. The reasons for this is unknown. The dynamic expansion of proteins domains within composite proteins, especially related to extracellular processes, took place in the eukaryotes [61]. One can thus speculate that if multiple vWFA domain duplication events and incorporation into different proteins occurred over a relatively short period of time, for instance with the earliest eukaryotes and perhaps later as the invertebrate chordate line led to the vertebrates, that the similar degree of differences among groups of vWFA domains may make it impossible to resolve the relationships among them because they are all fairly equidistant from each other. Or perhaps the αI domain has arisen from duplication of the βI domain itself, but this appears not to be the case since the βI domain is among the most dissimilar of the vWFA domains in comparison of the αI domain (Fig. 1.6). The vWFA domain is small and even within the closely related human α subunits with I domains, or consider Ciα1 in Fig. 1.6, differences in some branching orders may arise if only the αI domain is compared rather than the full-length integrins having longer sequences and hence higher information content; this effect would likely be magnified when sequences are at even greater distances from each other. Whatever the reason, at the present there is insufficient information in the known sequences to resolve this issue. Interestingly, the sequence "Uncharacterized Protein 2" clusters closely with the ascidian αI domain as does one collagen IV α4 chain (Fig. 1.6).

1.7 A Summary of αI Domain Evolution and the Origin of the Vertebrate αI Domains

The integrin α and β subunits have a long history, likely originating in single-cell eukaryotes and thus predating the rise of the metazoans (Fig. 1.7). Despite the presence of homologues of most of the component domains of integrins within prokaryotes, integrins subunits have not been detected in bacteria. Integrins have also not been identified in non-metazoan multicellular organisms, namely fungi and plants (despite attributing a small protein from *Arabidopsis thaliana* as an integrin-like protein rather than possibly a fibronectin-like domain that might be *recognized by an integrin*; [49].

The earliest observed integrin subunits are in single cell eukaryotes diverging close to the origin of the metazoans [79]. Already in *C. owczarzaki* multiple integrin α and β subunits are observed and this is true for the first metazoans too, e.g. *T. adhaerens* and *A. queenslandica*, suggesting that integrin function assumed multiple roles from the very beginning. In a single species, integrin α subunits of humans frequently outnumber β subunits and thus a single β subunit may have multiple α subunit partners; in humans, for example, the β1 subunit forms dimers with 12 of 18 α subunits.

From the earliest integrins, ligand binding (e.g. [98]) was likely based on interactions at MIDAS of the βI domain and with the β propeller domain of the α subunit. The proteins ligands recognized by these early integrins likely also had short recognition signatures, e.g. RGD, LVD and variants, that were presented on surface loops that could occupy the fairly narrow site between the β propeller of the α subunit and the βI domain of the β subunit. The integrin domain structure then remained quite static in terms of domain structure throughout the invertebrates and into the first invertebrate deuterostomes, e.g. the echinoderms. This is also

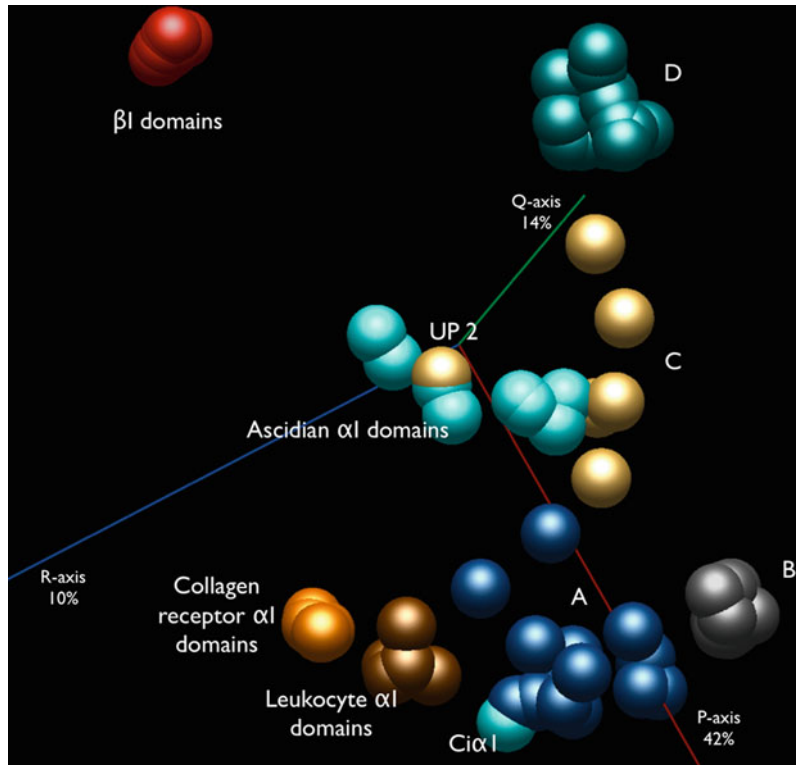

Fig. 1.6 Multivariate analysis showing the mutual relationships among integrin αI and βI domains from human and the ascidians, and other vWFA domains. The integrin βI domains from human and the ascidians cluster together and are the most dissimilar cluster from all others. Ciα1 clusters with a group A, whereas UP2 (Uncharacterized Protein 2) clusters with the Ciona αI domains. *A* Chains from collagen type VI, XII, XXII, UP1, Sushi 2–3, Fibrillins, Cartilage matrix protein. *B* Chains from collagen type XIV, XII; Selectins (E,F,P); Matrillin, Sushi 1,3; Fibrillins; UP3-6; *C* Collagen type VI chain, Anthrax toxin receptor 1, Plasmodium CTRP, Plasmodium micronemal protein. *D* Calcium channels; Trypsin inhibitors; Copines; Midasin: Bacterial Mg^{2+} chelatase; Yeast DNA repair factor; Yeast Proteosome regulatory factor). The comparisons and figure were generated as in Fig. 1.5

true for the lancelets. The cephalochordates are now accepted to have diverged prior to the tunicates and are the earliest diverging extant chordate subphylum.

In an ancestor of the urochordates, a key change in an integrin α subunit gene led to a major alteration of the protein structure and function of some integrin heterodimers: A vWFA-type domain was inserted within a loop at the surface of the β-propeller domain of the α subunit. The urochordates remain the earliest diverging extant species found to have αI domains, with one in *H. roretzi* and eight in *C. intestinalis*. The nine integrins α subunits with I domains for their own clade are clearly not of the collagen or leukocyte types seen in humans and other vertebrates. The α1 I domain of *C. intestinalis* has been tested for collagen binding and, unlike the human collagen binding set, does not bind to fibril forming collagens I–V nor to GFOGER-like peptides, but it does bind strongly to collagen IX through a mechanism that is metal and MIDAS independent [93]. MIDAS-independent binding occurs with other vWFA domains: e.g. vWFA3 [7, 35, 74], known to bind collagen I and III, and vWFA1 [13], which binds platelet glycoprotein 1b alpha.

The αI domain thus appeared early in chordate evolution and within an invertebrate. This inserted domain relocated the integrin external

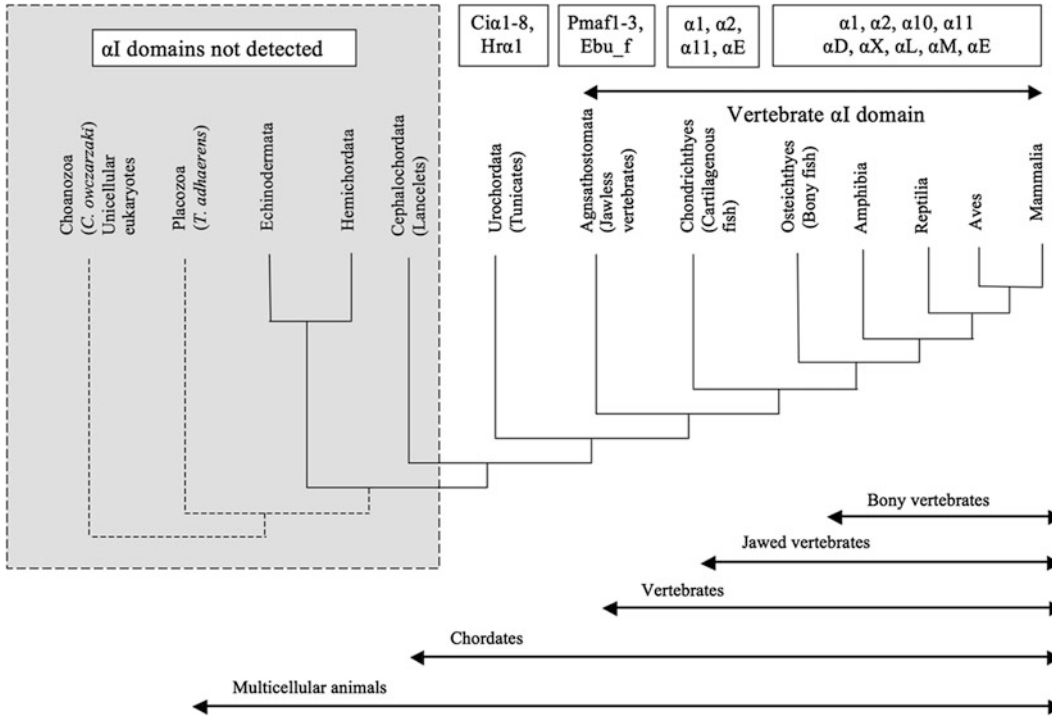

Fig. 1.7 The spectrum of identified integrin and αI domain sequences. The earliest diverging species with identifiable integrin sequences are in the single-cell eukaryotes, phylum choanozoa, e.g. *C. owczarzaki*. Multiple α and β subunits found in the choanozoan are typically found in the metazoans and the number of α and β subunits generally increases for species with later divergence times too, and especially within the chordates. βI domains are an essential part of the heterodimeric structure and are found in all β subunits. αI domains have not been detected among non-deuterostome invertebrates, nor in echinoderms and the earliest diverging chordate invertebrate, the lancelet. The earliest diverging species having integrin α subunits with I domains are the invertebrate urochordates, but these α subunits form a clade distinct from the αI domains found in the vertebrates. Fragments of α subunits with I domains are found in lamprey and possibly hagfish and they appear most similar to sequences from the collagen-binding group and the leukocyte group found in other vertebrates, including the elephant shark, bony fish and other vertebrates through to humans

ligand binding site away from the β propeller—βI domain interfacial cleft to location on the αI domain, which itself budded out from the β propeller. The αI domain in humans make use of a key feature of many vWFA domains, including the βI domain; that is, MIDAS for binding negatively-charged amino acids at the positively-charged metal ion located at the site. But, it is not clear what mechanisms are employed by the ascidian tunicate αI domains for ligand recognition. Nevertheless, MIDAS is conserved and the majority of the known ascidian sequences contain the intrinsic glutamate ligand involved in triggering the receptor activation mechanism (Fig. 1.3). A major advantage of the αI domain in integrins is high solvent exposure near MIDAS, meaning that recognized proteins no longer needed flexible loop regions to snake into a binding cleft. Instead, larger and more bulky surfaces and fibrils could now directly interact with the integrins. Thus, we see that glutamate within collagen-like triple helical peptides bind to MIDAS and, similarly for ICAMs, glutamate extends from the end of a beta-strand at the immunoglobulin fold surface and binds to MIDAS at the αI domain.

In humans there are nine integrin α subunits with I domains; four are within the collagen-binding set and five belong to the leukocyte-specific clade. Orthologues extend across the

mammals, birds, reptiles, amphibians, and bony fish—the latter often having duplicate isoforms. The cartilaginous fish also have orthologues of human-type α subunits with I domains. Preliminary data now exist that the earliest diverging extant vertebrates have human-like integrins with αI domains too, since lamprey and perhaps hagfish appear to have integrin αI sequences that are respectively most similar to the collagen binding (i.e. α1, α2, α10 and α11) and immune cell recognizing (i.e. αD, αE, αL, αM, αE) integrin α subunits.

The I domain in the integrin α subunit may have helped to facilitate or accommodate the large scale changes and stresses that accompanied the rise and diversification of chordates, and the added complications of expanded interrelated physiological systems and specific functional organs and tissues. The incorporation of the αI domains into some α subunits, no matter how this occurred, did provide the chordates with a broad spectrum of tools for recognizing the extracellular matrix and other cell types, and allowed cells to deal with a wider range of complications associated with the multicellular complexity of the rapidly expanding vertebrate line.

A key defining feature of the vertebrates is cartilage and bone, and collagen receptors with high avidity may have been necessary for their development as well as for other tissues [31]. The skeletal system is also tightly interconnected to the immune and circulatory system, two other systems where major changes also took place during chordate and especially vertebrate evolution. In the cyclostomes, e.g. lamprey, the circulatory system has fibrinogen-based blood clotting [23], and integrins with αI domains are key receptors involved in e.g. vertebrate platelet aggregation. The vertebrate adaptive immune system relies heavily on I domain containing integrins. In the ascidian H. roretzi, αHr1 is associated with the innate complement system of defense, whereas lamprey and hagfish had developed a unique adaptive immune system preceding that seen in higher vertebrates [66, 106, 46]. The interrelationships between these systems for support, defense, and mediation of nutrition and waste removal from distant tissues would have benefited from a expanded functional set of both classes of vertebrate integrins with αI domains that could recognise a wider array of ECM ligands and cell-surface receptors.

Finally, we can state with relative certainty based on the data now at hand, that [1] the αI domain originated early in chordate evolution but after the divergence of the lancelets and in an common ancestor of the tunicate and the vertebrate lines. We can also state that [2] specialization of α subunits with I domains towards collagen recognition and leukocyte binding took place soon thereafter as reflected in preliminary data from the lamprey and hagfish genomes; and [3] already within the cartilaginous fish several orthologues of the human type integrin α subunits with I domains are identifiable and thus, given the range of observations, the collagen-binding integrin clade and the leukocyte clade are characteristics not just of humans and other later diverging vertebrates, but of the vertebrates as a whole (Fig. 1.7) including the agnathostomes—they are the vertebrate αI domains.

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Integrin $\alpha 1\beta 1$

2

Humphrey Gardner

Abstract

Integrin $\alpha 1\beta 1$ is widely expressed in mesenchyme and the immune system, as well as a minority of epithelial tissues. Signaling through $\alpha 1$ contributes to the regulation of extracellular matrix composition, in addition to supplying in some tissues a proliferative and survival signal that appears to be unique among the collagen binding integrins. $\alpha 1$ provides a tissue retention function for cells of the immune system including monocytes and T cells, where it also contributes to their long-term survival, providing for peripheral T cell memory, and contributing to diseases of autoimmunity. The viability of $\alpha 1$ null mice, as well as the generation of therapeutic monoclonal antibodies against this molecule, have enabled studies of the role of $\alpha 1$ in a wide range of pathophysiological circumstances. The immune functions of $\alpha 1$ make it a rational therapeutic target.

Keywords

Integrin · Collagen · Knockout mouse · Phenotype

2.1 Introduction

The integrin $\alpha 1$ subunit was first discovered by Hemler et al. as the α component of the Very Late Antigen I (VLA1) expressed on a subset of T cells in the joints of patients with rheumatoid arthritis [57], as well as in a subset of lymphocytes after long term in vitro culture. $\alpha 1$ is the largest of the α subunits, with an apparent mw of 190 kDa nonreduced and 210 kDa reduced [60]. $\alpha 1$'s larger size compared to $\alpha 2$ is due to a higher degree of glycosylation [59]. At the C terminus, the intracellular portion of $\alpha 1$ is the shortest of the α subunits, at 13 residues. Functionally, $\alpha 1$ is one of four collagen binding I-domain containing $\beta 1$ partners, along with $\alpha 2$, $\alpha 10$ and $\alpha 11$. None of the four are known to partner with any β subunit other than $\beta 1$. The $\alpha 1$ I domain shows, like $\alpha 2$, 10 and 11, affinity modulation of ligand binding activity in the same way as has been described for αL [89, 133].

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2.2 Tissue Distribution and Gene Regulation

$\alpha1\beta1$, like $\alpha11\beta1$, is predominantly present in mesenchyme. In the adult $\alpha1$ is most abundant in vascular and visceral smooth muscle. This smooth muscle expression has been shown, in the chicken, to be due to a unique combination of transcription factors, GATA6, SRF, and Nkx3.2 [101]. The latter is not found in mammals, but similar factors such as Bapx1 and its family members may play the same role. $\alpha1$ expression is switched off during megakaryocytic differentiation and this appears to be due to gene methylation [20]. The regulation of $\alpha1$ baseline expression in other tissues has not been extensively explored. Other sites of $\alpha1$ expression include fibroblasts [136, 142] and, particularly, specialized fibroblast related cells such as hepatic stellate (Ito) cells [112], pericytes [142] and mesangial cells [60, 96]; bone marrow mesenchymal stem cells [36, 54]; chondrocytes [85], in concert with integrin $\alpha10$ [18] and $\alpha2$ [152]; neural cells including undifferentiated Schwann cells [139] and neurons [37]; and many white blood cells [44, 59]. Microvascular endothelium shows abundant $\alpha1$ expression [33], which is upregulated during angiogenesis. Surprisingly, immunoelectron microscopy shows the presence of abundant $\alpha1\beta1$ on the luminal, as well as abluminal, endothelial surface [16], where no canonical $\alpha1$ ligand would be expected to be. $\alpha1$ is generally absent from normal epithelia, other than the endoderm derived hepatocytes [55, 86, 137], retinal pigment epithelium [99], and endometrial glands [10], where it is cyclically expressed.

Although SNPs in *ITGA1* have been associated with osteoporosis in Korean populations [80], these are synonymous and do not have associated expression data to corroborate their relevance.

2.3 Expression During Development

During development, there is abundant and dynamic expression of $\alpha1$ in embryonic tissues. It is first seen at the leading edge of invading trophoblast shortly after implantation [140], and antibody blockade of $\alpha1$ inhibits trophoblast invasion in vitro [32]. During early to mid embryogenesis $\alpha1$ is expressed transiently by neurons of the CNS [37], by maturing skeletal and cardiac muscle [144], in the skin [61], throughout the developing kidney [73], and in neural crest cells as they mature to dorsal root ganglia [37].

2.4 Expression in Malignancy

Dysregulation of $\alpha1$ has been noted in tumors. Some studies of melanoma have shown a correlation of worse clinical behavior with the presence of $\alpha1$ [124, 125], and others with the absence of $\alpha1$ [50]. Leiomyosarcomas often show loss of $\alpha1$ and gain of $\alpha2$ [94]. Bronchoalveolar [75] and gastric [46] carcinomas sometimes show gain of $\alpha1$ expression, as do squamous cell carcinomas of the head and neck [114]. Survey of RNAseq signatures of the GATC database shows that $\alpha1$ is in general reduced in total expression in tumors compared to normal tissues, probably reflecting the increased epithelial to mesenchyme ratio of the tumors, whereas the reverse is seen for the more epithelially expressed $\alpha2$ (Fig. 2.1). The two exceptions to this finding are head and neck SCC, corroborating Ratzinger et al. [114], and clear cell carcinoma of the kidney (Fig. 2.1). Lastly, dermatotrophic T cell lymphomas show expression of $\alpha1$ [138] probably consistent with the ontogeny of their derivation in the T cell lineage. There is no consistent relationship between $\alpha1$ expression and tumor behavior, in contrast to, the well-characterized and functionally significant $\alpha6\beta4$ to $\alpha6\beta1$ switch seen in some epithelial malignancies.

2.5 Integrin $\alpha1$ Ligands

The best-known ligands for $\alpha1$ are the collagens, investigated mostly in fibroblast studies, and laminin 111, investigated primarily in studies of neural cells. Other $\alpha1$ ligands include matrilin-1,

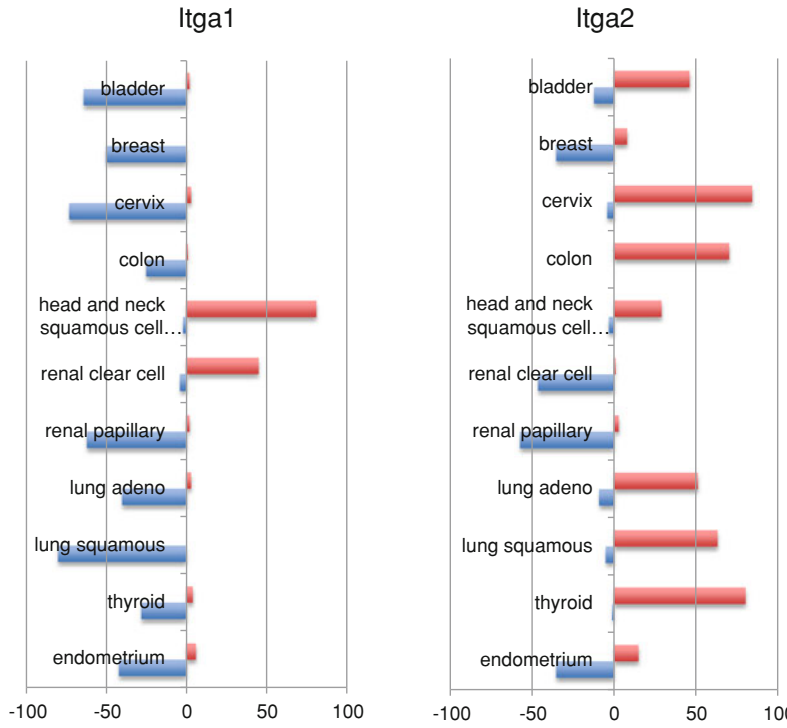

Fig. 2.1 Expression of Integrins alpha1 and 2 in different tumor types. Ratio of RNAseq counts for the gene in tumor versus matched normal was determined. Data taken from TCGA where total evaluable number of samples for the tumor type exceeded 100. Numbers of samples where the ratio of expression exceeded 2 were quantitated. *Red bars* indicate the proportion of cases where tumor expression is twofold or more greater than matched normal, and *blue bars* where tumor expression is twofold or more lower than matched normal. With the exception of renal tumors, Itga2 tended to be increased in expression in tumors in comparison to normal tissue. With the marked exception of clear cell carcinoma of the kidney and head and neck squamous cell carcinoma, Itga1 tended to be downregulated in tumors versus normal tissue

expressed in cartilage, galectins 1, 3 and 8, and the NC1 domain of collagen IV(1), which will be discussed in the context of endothelial regulation. Lastly, semaphorin 7A expressed on macrophages appears to be a counterreceptor to α1β1 [141]. Ligands are listed in Table 2.1.

2.5.1 Collagens

α1 and α2 β1 integrins have collagen binding preferences that are at first glance discordant with their tissue distributions. α1β1, predominantly expressed on connective tissue, has a higher affinity for collagen type IV than for type I; whereas α2β1, predominantly on epithelial cells, favors collagen I, which epithelial cells do not normally see, over the collagen IV abundant in epithelial basement membranes. α1 and α2 (and probably α10 and α11) bind the triple helical domains of the collagens with highest affinity, and biochemical, cell biological and crystallographic studies show that this binding is contributed to by more than one chain of the triple helix [39, 42]. As such, the binding is dependent on the chains being in register, and would thus be exquisitely sensitive to melting. As collagen melting occur at or below physiological temperatures in a very dynamic fashion [81], it is likely that α1 ligand binding, and hence signaling, can be affected by events distal to the receptor along the collagen fibril. This might be especially important in tissue remodeling.

The α1 I domain can bind the collagen triple helix at multiple different sites [117, 153], with the relative affinities being divisible into several

Table 2.1 Known ligands of Integrin $\alpha1\beta1$

Ligand	Likely cellular context	References
Collagen I	Fibroblasts	[39]
Collagen IV	Fibroblasts, myoepithelium	[39]
Collagen IX	Cartilage	[76]
Collagen XVI	Connetctive tissue	[40]
Arresten (Col4A1 NC1 domain)	Angiogenesis	[25]
Laminin 111	Neural tissue	[143]
Laminin 112	Neural tissue	[143]
Matrilin I	Cartilage	[91]
Galectin 8	T cells	[31]
Galectins 1, 3	Vascular smooth muscle	[98]
Jararhagin	Snake venom	[104]
Obtustatin	Snake venom	[92]
Ross River Virus	Viral infection	[82]
Semaphorin 7A	T cell macrophage interactions	[141]

classes. Among these there are approximately three of the highest binding affinity, with Kds of ~0.25 uM, and about 13 in the next affinity class, with Kds of ~14 uM. The highest binding class regions are adjacent to or overlap with the sites occupied by α2 I domain, and these can be competed off both α1 and α2 I domains by triple helical model molecules containing the core sequence GLOGER or GFOGER, the latter of which was also independently identified as an inhibitor of α1 and α2 binding to collagen I [72] as well as α11 [158]. This core peptide is not effective in blocking α1 binding to collagen IV, but is effective in blocking α2. Recently the peptide GFPGEN was identified as a sequence selective for binding α1 over α2 [130]. The collagen IV binding site for α1 is unique and of higher affinity [17], and has been shown by to require Asp 461 in the α1 chain of collagen IV and Arg 461 in the α2 chain [39]. The binding of integrin α1, α2 and α10 I domains to other collagens has also been explored [103, 147]. More recently α1 has been clearly identified as a receptor for the FACIT collagens IX (predominantly in cartilage) [76] and XVI (predominantly in connective tissue) [40], in a region close to that bound by α2. Mutation of Arg 218 to Asp in α1 causes loss of collagen IV and IX binding, but only partial reduction in collagen I binding [76]. Structural analysis based on modeling from the α2 subunit demonstrates the existence of closed and open states alternately blocking or enabling binding of RKKH type peptides. The two states are energetically very similar, allowing for the possibility of control by inside-out signaling [104]. Another mutation in α1, Glu 317 to Ala, causes increased affinity of the I domain for both collagens and laminin [146], and reveals the possibility that the activated integrin, and ligand bound open integrin, may be slightly different states [77]. Dramatically, this I domain mutation Glu 317 to Ala also causes increased activation of ERK, and enhanced downregulation of collagen synthesis [132], further affirming outside–in signaling and attributing it to the integrin itself. The relationship between the probable affinity modulation of α1, the multiplicity of sites on the collagen fibril along which α1 can bind, and the dynamic instability of the triple helix, suggest a highly dynamic interaction between integrin and collagen. For example, one could see how fibroblast motility along collagen I might be contributed to by detachment and reattachment of the integrin along the fibril. Another possibility is that collagen fibril assembly and extrusion from the fibroblast might be aided by $\alpha1\beta1$ protruding from the plasmamembrane

surrounding the fibril. Indeed, the α1 null mouse has narrower and less well formed collagen fibrils than the wild type animal (Gardner, unpublished). However, the relative importance of α1 binding to collagen I versus the basement membrane and facit collagens in vivo has not been established.

2.5.2 Laminins

Laminin 111 and 211 binding by α1 is seen in fibroblasts, and is particularly evident on neural cells, for which the pheochromocytoma line PC12 is used as a prototype [143, 159]. These cells show α1 dependent adhesion to domain VI of the laminin α chains 1 and 2, at sites adjacent to or congruent with α2, at the opposite end of the laminin molecule from the binding regions of the epithelial laminin receptor α3β1 and the hemidesmosome integrin α6β4 [24]. This seems reasonable in the context of an epithelial basement membrane, where epithelial cells would bind at one end of the molecule and mesenchymal cells at the other (although binding to laminin 332 by α1 is not seen). In vitro, α1 has been [13] found to be important for neurite outgrowth on laminin [145] and neural crest cell attachment to collagen [108]. Neural crest cell attachment to laminin can be inhibited by antisense oligonucleotides to α1 mRNA [78]. Further studies have shown that neural crest cells migrating on laminin 111 interact, via α1, with two distinct sites on the molecule. LN E8—α1 interaction drives FAK activation, focal adhesion formation, and migration, while LN E1—α1 interaction drives ERK activation and survival [35]. While it is tempting to suggest that this specificity is attributable to subtleties of outside in signaling, the work does not rule out the possibility of some essential coreceptor for one or other interaction. The α1 null mouse, however, has normal pigmentation on all genetic backgrounds and appears neurologically and neuroanatomically normal except for a sensitivity to ketamine/xylazine anesthesia (Davidson J, unpublished observations) which may have a neurological basis. Whether α2, possibly co-expressed on neurons, provides an adequate alternative for neurite outgrowth, will be seen in the α1/α2 double null animal.

2.5.3 Matrilin and Galectin

Matrilin-1 is found in cartilage, and appears to cause increased chondrocyte adhesion to collagen II, via its association with α1 [91]. Galectin 8 [31] binds several integrins including α1 but not α2, and induces Erk phosphorylation independently of cell attachment. Galectins 1 and 3, secreted by vascular smooth muscle, also appear to bind integrin α1, the latter in a lactose dependent manner [98]. These glycoproteins, in contrast to matrilin-1, appear to inhibit cell attachment to other matrix components.

2.5.4 Semaphorin 7A

The semaphorins are best known as guidance molecules in the CNS. Interestingly, Sema7A, a subset of semaphorins primarily found in the immune system, appears also to be a component of the immunological synapse in some activated T cells [141], where it interacts specifically with macrophages expressing integrin α1β1, inducing downstream effects of α1 activation. Similarly to α1 null mice, sema7A null animals are resistant to encephalitis and DTH models. α1β1 is widely expressed in the CNS. Whether it interacts with other semaphorins is to be seen.

2.6 Peptide Inhibitors of α1

While Jararhagin, a venom protein first noted to bind the alpha2 I domain, also binds the α1 I domain [104], Marcinkiewicz and colleagues also identified Obtustatin [92] as a specific inhibitor of α1 which does not bind to the I domain. Using blockade of FGF2 driven angiogenesis in the chick CAM model as an assay, they pinned down a specific inhibitory peptide

with affinities in the millimolar range, with sequence CWKTSLTSHYC. No further work has been published on this interesting molecule.

2.7 Co-receptors of α1

Many non-I domain containing integrins have been shown to associate in the membrane with other receptors, the best examples being the tetraspanins [8] and Integrin Associated Protein [15]. These may modulate integrin behavior and binding to ligands. α1 has not been shown to associate with such proteins, but this is an area meriting further exploration. On the other hand, α1 is one of a subset of integrins (including α5β1, αvβ3, and α6β4) which associate in the membrane with caveolin and stimulate the Erk pathway via Fyn and Shc [150, 151].

2.8 Integrin α1 Regulation by Cytokines

Most studies of regulation of α1 expression in the adult relate to expression during lymphocyte ontogeny, and in fibroblasts in response to a variety of cytokines. Like integrins α2, α3, α4, and α5β1, α1β1 is upregulated in fibroblast lineages by TGF-β [56], as well as interleukin-1β [123], TNF-α, and interferon gamma [47]. The only cytokine which appears to cause differential regulation of α1 is platelet derived growth factor—BB, which causes downregulation of α1 integrin and upregulation of α5 integrin in fibroblasts [47] and mesangial cells [68].

2.9 The α1 Null Mouse

Aspects of the α1 null mouse will be discussed in subsequent sections. A brief overview is provided here to provide perspective on the known and suspected roles of α1. α1 null mice are viable and fertile, and embryogenesis proceeds normally despite the broad and dynamic expression in trophoblast and developing nervous system. Initially, adult animals are remarkably normal with a mild decrease in weight, normal smooth muscle function, normal rates of wound healing, normal liver function, normal behavior, and no blatant immunodeficiency in a laboratory environment [48]. With ageing, the animals exhibit a series of progressive phenotypes, notably osteoarthritis [156], and retinal degeneration [107], as well as a variety of other vulnerabilities.

2.10 Integrin α1, Signaling, and the Cell Cycle

The potential role of α1 as a cell cycle regulator was suggested by studies showing that α1β1 was a member of a small group of integrins which could activate the adaptor protein Shc, resulting ultimately in MAP kinase activation [150]. Several observations from the α1 null mouse confirmed this, including a reduction in fibroblast proliferation rate in embryonic skin and dermal fibroblast number in the adult, as well as the observations that embryonic fibroblasts from the α1 null failed to activate Shc in response to adhesion to collagen, and that they failed to grow on collagen in conditions of limiting serum whereas growth on the α5 ligand fibronectin or the αv ligand fibrinogen, was normal [109]. As α2 and probably α11 are present on these cells, this suggests that α1 is unique among collagen binding integrins in mesenchyme in being able to stimulate proliferation. Fracture calluses are smaller in α1 null mice, concomitant with a deficiency in bone marrow derived mesenchymal stem cell proliferation [41]. Interestingly, the number and proliferation of mesenchymal stem cell derived hypertrophic chondrocytes in this model is normal—suggesting a specific and transient dependence on α1 for proliferation in the mesenchymal stem cell differentiation pathway. Indeed, α1 has been identified as a very effective tool for the isolation of mesenchymal stem cells [36], and more recently for the selection of the most proliferative subclones of mesenchymal stem cells with the highest multidifferentiation potential [120]. A role for α1 has also been described in osteoblast differentiation [66]. Similar phases of α1 dependence for

proliferation appear to be present at some stages of lymphocyte ontogeny [95]. Furthermore, tumor cells derived from Kras transgenic mice are less proliferative on an α1 null background [90]. Overall, the subtlety of the proliferative deficit in the α1 null mouse must be accounted for by the large number of overlapping proliferative pathways, ligands, and integrins present in the organism.

The α1 cytoplasmic domain is very short. It is required for α1β1 migration into focal adhesions [12], and has a role in binding cytoskeletal components [87, p. 125] FAK, and phospholipase C gamma [149]. A remarkable study by Abair et al. [1], taking advantage of α1 null endothelial cells, demonstrated very specific requirements of components of the tail for full activity. The lysine triplet is required for migration and adhesion, and for activation of the Akt and p38 pathways, but not for Erk activation. Furthermore, alanine scanning shows that the most membrane proximal lysine is required for endothelial tubulogenesis, and migration on collagen IV, and that Lys 1151 is required for all functions except for proliferation. It appears that the integrin α1 cytoplasmic tail is quite unique among the integrins in being able to bind and activate the small nuclear shuttling phosphatase TCPTP. This phosphatase has many targets, but in the context of collagen ligand binding, TCPTP acts to cause a reduction in EGFR signaling [93], either by dephosphorylating EGFR directly or by reducing the amount of phosphorylated caveolin available to activate EGFR [11]. Whatever the mechanisms, the implication that active ligand binding, which in general would cause Erk activation, can serve to dampen an alternative pro-mitotic signaling pathway is intriguing. The specificity to α1 is also intriguing. While the genomic region containing α1 is lost in some tumors, and thus α1 might be regarded as a candidate tumor suppressor [93], the molecule is not expressed in most epithelial tissues. One physiological site where α1 might usefully downregulate EGFR activity is in myoepithelial cells of the breast, where cells express α1 [74], as well as EGFR [100], and are juxtaposed to basement membrane.

2.11 Integrin α1, Fibroblasts, and Collagen and Collagenase Regulation

Many studies have shown that α1β1 is a negative feedback regulator of collagen synthesis by fibroblasts. These were initiated by Langholtz et al., who showed that an activating antibody to α1 accentuated the normal downregulation of collagen synthesis seen when fibroblasts are suspended in collagen gels [79]. It was also noted that α1 levels appeared to be reduced on scleroderma fibroblasts, in conjunction with their upregulation of collagen synthesis [64]. Data from the α1 null mouse lent strong support to this role: in vivo the mice show a 20 % increase in the rate of collagen incorporation into the skin, and fibroblasts from these animals are deficient in downregulating synthesis in response to gel suspension [49]. We subsequently examined keloids to determine whether loss of α1 could account for the increased collagen expression in these lesions [142]. A high proportion of lesional fibroblasts expressed α1 (in contrast to scleroderma lesions), although the levels expressed were somewhat lower than seen in chronic wounds with low collagen production. Thus, absence of α1 could not account for the excess collagen production in keloids, but there may be a relative deficiency compared to normal wounds, which show distinct peaks in α1 expression at 8 and 30 days [9].

The mechanism for downregulation of collagen synthesis mediated by α1 has been extensively dissected. α1β1 stimulation by ligand activates the MAP kinases Erk1 and 2 via Fyn and Shc [109, 150], and Erk1/2 activation reduces collagen synthesis [116]. Reciprocally, the Erk1/2 inhibitor PD98059 causes upregulation of fibroblast collagen synthesis [1]. This is the reverse of the effect of α2β1 stimulation, which activates p38 and causes induction of collagen synthesis [65]. Thus, in general, α1β1 and α2β1 are opponents in their effect on collagen synthesis, the former inhibitory and the latter activatory. More specific mechanisms of collagen regulation involving reactive oxygen

species in mesangial cells will be discussed in the kidney chapter.

The regulation of metalloproteases has similar themes but appears more complex and is probably very cell type specific. Firstly, the structure and function of the mouse and human collagenases is not congruent: MMP1, which is the major fibroblast collagenase in humans, is upregulated by Erk1/2 activation, but the mouse MMP1 structural equivalents, McolA and McolB, are not seen in skin fibroblasts, and have a restricted expression in the placenta and uterus [3]. On the other hand human MMP13, found in chronic ulcers [148], is downregulated by Erk1/2 activation as well as being upregulated by p38 activation [115], and MMP13 is the major fibroblast collagenase in mouse [83]. Although functionally equivalent to human MMP1, mouse MMP13 appears to be regulated like human MMP13, as it is markedly upregulated in α1 null mice where there is loss of Erk1/2 signaling but normal α2-p38 signaling. The α1 null animal shows an increase in expression of several other MMPs, including 7, 9 and 2 in endothelial cells, and 9 and 2 in fibroblasts [49, 111]. For want of other evidence, this may be attributed to reduced Erk1/2 activation. However, whereas α1 stimulation is always inhibitory to collagen synthesis, it is sometimes activatory to MMP synthesis. In some systems α1 activation by laminin [84] or by collagen IV (Pozzi and Gardner, unpublished) or collagen I [121] causes an increase in MMP synthesis.

In many studies of fibroblast collagen interaction, the complex process of collagen gel contraction is addressed. In dermal fibroblasts integrin $\alpha 2\beta 1$ is seen to be the dominant player in this process [79], which can be uncoupled from MMP synthesis [14], and is dependent on a functional cytoskeleton. However, in studies of specialized cardiac fibroblasts [19], smooth muscle cells [53], stellate cells [113] and mesangial cells [69], α1 blockade has been shown to prevent gel contraction, as has integrin $\alpha v\beta 3$ blockade in other cell types [27]. It is possible that whereas α2 is structurally more suited to gel contraction (having a far higher affinity for collagen I), α1 expression may be required for maintenance of the contractile myofibroblastoid phenotype. It is striking that α1 expression is upregulated in vivo in all activated contractile myofibroblastoid cells including myofibroblasts in wound repair, mesangial cells, pericytes, myoepithelial cells [74], and hepatic stellate cells.

In summary, there may be several roles for α1 and its interplay with α2 in the fibroblast during dermal wound healing and other episodes of mesenchymal repair. α1 upregulation in fibroblasts contributes to collagen stimulated cell proliferation, and probably to the myofibroblast transition. α2 is the major contributor to the synthetic phenotype, where it contributes the major part of collagen matrix contraction and activates collagen synthesis, as well as activating MMP synthesis for matrix remodeling. α1 fine tunes the MMP response, possibly providing general inhibition of MMP release, but allowing for specific activation near the epidermal boundary where there is a greater abundance of the α1 high affinity ligand, collagen IV. α1 also provides feedback inhibition against excessive collagen synthesis. Consistent with these suggestions, the α1 null shows excessive collagen and collagenase synthesis at overlapping phases of wound healing [49], and collagen fibrils are densely aggregated and irregular in the dermis of the α1 null, while being individually smaller (Gardner, unpublished observations). Some aspects of this paradigm appear to be different in mesangial cells, which are discussed in the kidney chapter.

2.12 Integrin α1 and Angiogenesis

Immunohistochemical analysis of murine and human tissue shows that α1 is present on at least some normal microvascular endothelium. α1 has also been shown to be upregulated on endothelia in MS lesions [135]. New tumor microvessels appear always to express α1, while a smaller proportion of them, predominantly the slightly larger ones, also express α2 [111]. Vascular endothelial growth factor (VEGF/VPF) can induce α1 on endothelial cells, and as the only

collagen receptors expressed, α1 and α2 are required for endothelial haptotaxis through collagen. Antibodies to α1 and α2 reduce angiogenesis in response to subcutaneously implanted gels of fibrin or collagen containing VEGF, or to tumor xenografts [128, 129]. However, tumor matrix contains a great variety of alternative integrin ligands. As we have learned from fibroblasts, α1β1 can activate an Erk1/2 proliferation pathway mediated by Shc. α2β1 can also positively regulate the progression through the cell cycle in epithelial cells by non overlapping mechanisms [71]. Thus, α1 and α2 blockade in vivo may cause a simple reduction in endothelial proliferation. With this in mind, there is no deficiency in normal vasculo- and angiogenesis in α1 null mice. Analysis of the null mice, however, reveals other, subtler roles for α1 in angiogenesis.

Detailed analysis of endothelial cells and tumor vasculature in α1 null animals [111] led to independent verification of the significance of plasminogen fragments, the angiostatins [105], in endothelial growth regulation. Pulmonary microvascular endothelial cells from α1 null mice grew poorly compared to wild type, regardless of the substratum on which they were grown. This growth deficiency could be completely rescued by frequent media change even if the cells were grown on collagen. Furthermore, media conditioned by α1 null endothelial cells was inhibitory to the growth of wild type cells. The growth deficiency in α1 null endothelial cells was also corrected by antibodies to angiostatin, or growth in media containing serum from plasminogen null mice (from which no angiostatin could be generated) instead of fetal calf serum. Lastly, the growth deficiency could be rescued by MMP9 blockade. Analysis of conditioned medium from α1 null endothelial cells as well as plasma from wounded (but not unwounded) or tumor bearing α1 null mice showed an increase in MMP9 and angiostatin compared to wild type animals. These findings in endothelial cells were consistent with the increased MMP expression seen in α1 null fibroblasts, due to loss of α1-Erk1/2 inhibitory signaling with normal α2-p38 activatory signaling. Thus, increased MMP9 released by the α1 null cells cleaves plasminogen [106] to yield the endothelial inhibitor, angiostatin.

In vivo, α1 null mice, with higher plasma MMP9 and angiostatin levels are less able to vascularize subcutaneous tumors than wild type, but this deficit can be reversed by oral treatment of the animals with the MMP9 inhibitor doxycycline, and consequent reduction of their angiostatin levels [110]. MMP9 levels in the vasculature correlate inversely with tumor vascularization even in wild type mice. These studies have been repeated in several tumor systems with essentially similar results, namely that tumors in the α1 null host are smaller and less vascular and the phenotype can be reversed by MMP inhibition [22, 23]. These studies showed that the interplay between α1 and α2 integrins has significant consequences in the vascular system. Thus, during vascular remodeling, upregulation of endothelial α1 and α2 occurs, and the balance between them regulates MMP release, and ultimately vessel number.

While plasminogen is an MMP9 target, and its cleavage product angiostatin was entirely responsible for endothelial growth inhibition in vitro, other MMP targets might be of importance in this feedback system in vivo. These include the collagens themselves. In this regard the finding that a collagen NC1 domain is a ligand for α1 may be of significance. The NC1 domain of collagen IV α3, also known as tumstatin, causes endothelial cytostasis and blocks angiogenesis by binding to integrin αvβ3, and a similar mechanism appears to exist for α1 binding to the collagen IV(1) NC1 domain (arresten) [25]. This might be an explanation for the presence of α1 on the luminal surface of endothelium, where it could act as a detector of collagen fragments released during remodeling, and provide negative feedback to angiogenesis. While in general α1 binding to collagen in fibroblasts causes activation of Erk1/2 via Shc, arresten may provide a growth inhibitory signal. In fact this has been strongly suggested by the work of Nyberg et al. where the arresten—α1 interaction appears to mediate an apoptotic response [102], and this interaction has been invoked in the blockade of

growth of HSC tongue carcinoma cells [2]. The absence of both signals—collagen IV driving growth and arresten being pro-apoptotic—in the α1 null could explain why normal angiogenesis is unaltered in α1 null mice.

2.13 Smooth Muscle and α1

α1 is extremely abundant on smooth muscle, both visceral and vascular [6], and, in vivo, expresses no other collagen binding integrin (explanted smooth muscle rapidly upregulates α2, complicating studies [134]). Furthermore, smooth muscle basal lamina has abundant collagen IV. There is no upregulation of α2 or α10 in the α1 null smooth muscle in vivo, as assessed by immunostaining [48]. Yet in the α1 null mouse digestion and parturition is entirely normal, and EM studies reveal no alterations in smooth muscle structure. Studies of mesenteric arteries have shown that α1 deficient vessels rupture at lower stresses than wild type, due to a deficiency in the hypertrophic response [88]. Integrin α8β1, a fibronectin receptor, is also abundant in smooth muscle, but the double knockout α1/α8 animal also had histologically normal smooth muscle (Gardner and Brandenburger, unpublished). Further collagen binding integrin double knockouts may reveal the answer to this mystery.

2.14 Integrin α1 and the Retina and CNS

Retinal pigment epithelium (REPE) cells have been shown to use α1 as one among other receptors for collagen gel contraction [99] but α1 signaling of MAP kinase activation is clearly of unique importance. Peng et al. [107] found that older α1 null mice become blind, with loss of retinal evoked potentials, degeneration of the peripheral retina, irregularities in basal lamina thickness, rod degeneration and synaptic malformation in rod and cone terminals, and failure of transducin α translocation to the outer rod segments upon light exposure.

Frasca et al. [45] have made observations on the role of α1 in contributing to the neurotoxicity of amyloid. This appears to be due to α1-ligand interaction, via Erk activation, being permissive to neuronal entry into the cell cycle after their stimulation by A-beta. Neurons, in contrast to other cell types, appear to meet their demise after cell cycle entry.

2.15 Integrin α1 as a Viral Receptor

Many integrins have been recognized as receptors for viruses. α1 appears to be one of several receptors for Ross River virus, a semliki forest type alphavirus one of whose coat proteins has a region which appears to mimic a collagen fold [82]. There is a possibility that α1 is also a receptor for rotavirus enterotoxin [131].

2.16 Integrin α1 and the Kidney

Expression of α1 by glomerular mesangial cells [30, 60] as well as the developing kidney [73] led to a great deal of interest in the role of this integrin in the kidney. α1 null mice showed no functional or anatomic renal abnormality alterations in α1 null glomeruli in the unperturbed state, but a variety of challenges have exploited the underlying mesangial alterations to create new models of renal disease. Ex vivo studies demonstrate alterations in mesangial homeostasis in the absence of α1, notably an alteration in MMP profile rather different from that seen in cutaneous α1 null fibroblasts [155]. α1 nulls also have poor osmolarity regulation [97]. Streptozocin treated α1 nulls get worse glomerular disease than wild type [157], and the diabetic Akita mouse gets dramatically accelerated renal dysfunction when crossed into an α1 null background [154].

Cross of the α1 null with the collagen IV α3 chain null (COL4A3/Alports) mouse [29] led to unexpected effects. Reduced glomerular basement membrane stiffness in the COL4A3 null leads to a progressive glomerulonephritis with mesangial expansion and secondary tubulointerstitial fibrosis. Surprisingly, the double null

animal lived twice as long as the COL4A3 null, due to a delay in the progression of renal failure. This unexpected result appears to be due to several mechanisms. Firstly, in the normal progression of murine Alports, there is a marked influx of monocytes into the interstitium in response to glomerular epithelial damage. α1 null monocytes are defective in migrating into the renal interstitium, possibly due to the monocyte requirement for α1 to adhere to the collagen XIII generated by endothelium during injury [34], and are therefore reduced in number in the double null kidney. This reduces delivery of TGFβ to the kidney, delaying the onset and progression of interstitial fibrosis [122]. Secondly, mesangial cells are dependent on α1 and Rac to invade the glomerular tuft [155], a key process in the initiation of renal repair and injury. In the α1/COL4A3 double null, the mesangial expansion is greatly reduced [29]. In another glomerulonephritis model, anti-Thy-1 GN in the rat [69], direct injection of anti-α1 in the renal artery caused a marked reduction in mesangial proliferation and matrix accumulation, an important in vivo validation of a series of studies of the role of α1 in mesangial cells [67–70]. The role of α1 in driving proliferation is complex in mesangial cells. In contrast to studies in most systems which ascribe a pro-proliferative role for α1 signaling, overexpression of α1 in mesangial cells leads to activation of p27Kip and cell cycle arrest [70]. In fact mesangial cells appear to be an exception in many aspects of α1 physiology, in that Erk phosphorylation is upregulated in α1 null mesangial cells and p38 is downregulated. Notwithstanding the increased Erk phosphorylation, collagen synthesis is increased, via a reactive oxygen species driven mechanism [21, 28]. This may be due to some kind of integrin crosstalk, where the excess integrin α1 activates a pathway normally associated with another integrin [127]. A potential corollary of this is that monomer and polymer collagen have different effects on mesangial cell growth; on the latter substrate growth is inhibited, α1 is excluded from focal contacts, and ERK1/2 phosphorylation is diminished [126].

2.17 Integrin α1 and the Immune System

Integrin α1 was first discovered as a very late antigen on cultured T cells, and being the largest of the α subunits, was named Very Late Antigen 1 (VLA1), a name which persists in immunological studies. Hemler et al. subsequently showed that VLA1 was present on a large proportion of T cells in the joints of rheumatoid arthritics, but was almost absent from the circulation, giving a first clue to a role for α1 in tissue migration and T cell activation [60, 58]. More detailed study of the immune system revealed that α1 is also expressed on a subset of NK-T cells as well as populations of activated monocytes and NK cells.

α1 deficiency generated by knockout or antibody blockade has dramatic consequences in the immune system. α1 null mice show no overt immunodeficiency, but they show resistance to many different disease models involving monocyte function or peripheral T cell memory. These include a resistance to anti collagen II antibody induced and mycobacterium induced arthritis [44, 62], colitis [43], DTH, contact hypersensitivity [44], and LCMV induced encephalopathy [7]. Inflamed tissues in these models, as well as the normal gut mucosal epithelium [95], show reduced infiltration by T cells and monocytes. Furthermore, cultured splenocytes from α1 null animals show reduced proliferation in response to collagen, and fail to express integrin α2 upon long-term culture.

In murine influenza models, α1 positive T cells tend to be CD4 and associate with basement membranes, while α2 T cells bias to CD4 and an interstitial location. Memory to influenza is maintained by the α1 positive T cells, as they are protected from TNF driven apoptosis [119, 118]. Treg cells are VLA1 negative, and stimulated PBMCs can be diverted from generating VLA1 + T effector cells into Treg cells if TNF signaling is blocked [51]. Taken together, the results suggest that α1 is needed both for lymphocyte migration into the collagen rich periphery, and for the proliferation of activated

T cells in those locations, or for their long term survival as mediators of peripheral T cell memory [38].

In rheumatoid arthritics, α1 positive T cells are far more abundant and tend to be found in the joints as oligoclonal populations, probably responding to a restricted number of joint antigens [4, 52]. Here they offer an obvious target for therapy. Interestingly, α1 has also been noted to be required for monocyte retention at sites of inflammation in skin [5], and a role for the receptor was similarly shown for T cells in a xenotransplantation model of psoriasis, where epidermal, but not dermal, T cells expressed α1 [26].

2.18 Therapeutics

In the early 2000s Biogen Idec developed a humanized function blocking anti-VLA1 antibody for immune diseases. This has now been taken through a phase 1 single dose escalation study by Santarus, as SAN-300, without remarkable side effects, and with anecdotal demonstration of efficacy in a single rheumatoid arthritis RA patient recruited to the study [63]. The potential for this molecule may be very high in diseases characterized by the persistence of localized pathological effector T cell memory, such as RA and psoriasis.

2.19 Summary and Prospects

Integrin α1 has major roles as a modulator of mesenchymal proliferation and differentiation, matrix turnover, and immune function. Its roles in the immune system make it a clear target for therapy. In its biochemical properties, α1 appears to have a unique role in binding basement membrane collagens, the significance of which in vivo is not yet entirely clear. Like the other collagen binding I domain containing integrins, α2, α10 and α11, its absence is not associated with major structural deficits in the mouse, illustrating the dense interweaving of redundant or partially redundant pathways in tissue morphogenesis.

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α2β1 Integrin

3

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Abstract

The α2β1 integrin, also known as VLA-2, GPIa-IIa, CD49b, was first identified as an extracellular matrix receptor for collagens and/or laminins [55, 56]. It is now recognized that the α2β1 integrin serves as a receptor for many matrix and nonmatrix molecules [35, 79, 128]. Extensive analyses have clearly elucidated the α2 I domain structural motifs required for ligand binding, and also defined distinct conformations that lead to inactive, partially active or highly active ligand binding [3, 37, 66, 123, 136, 137, 140]. The mechanisms by which the α2β1 integrin plays a critical role in platelet function and homeostasis have been carefully defined via in vitro and in vivo experiments [76, 104, 117, 125]. Genetic and epidemiologic studies have confirmed human physiology and disease states mediated by this receptor in immunity, cancer, and development [6, 20, 21, 32, 43, 90]. The role of the α2β1 integrin in these multiple complex biologic processes will be discussed in the chapter.

Keywords

α2β1 integrin · Collagen · Disease models

3.1 Collagen Receptors-Structure and Ligand Binding

The α2β1 integrin consists of an obligate heterodimer formed from the α2 integrin subunit non-covalently associated with the β1 subunit. It is one of four 'I domain' integrins, named for the presence of a highly conserved, extracellular, (inserted) I domain, which mediates specific binding of ligands including, most prominently, collagens [30]. The α2 subunit I domain is an autonomously folding domain of approximately 220 amino acids [30]. The I domain found in the collagen receptors is shared with the alpha subunits of the leukocyte β2 integrins and is highly homologous to the A domain found in Von Willebrand factor, in cartilage matrix protein, in some collagen subtypes and in components of the complement system. The crystal

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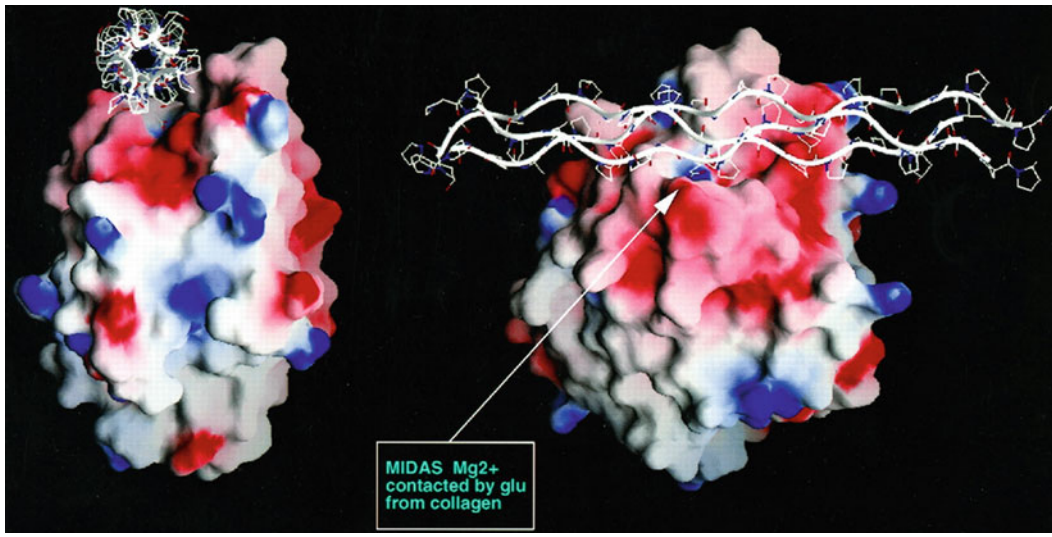

Fig. 3.1 A hypothetical model of an I-domain·collagen complex. A collagen triple helix (*white spiral*) is shown in a possible fit a groove on the MIDAS face. A glutamate side chain from the collagen coordinating the metal ion as indicated by arrow. The I domain is colored according to surface charge distribution (*blue* positive, *red* negative, *white* neutral. Two orthogonal views are shown (Reprinted from Fig. 5, Emsley et al. 1997)

structure of the α2 integrin I domain was first defined in 1997 (Fig. 3.1) [20]. The α2 subunit shares many similarities in structure and ligand binding with the other I domain integrins, including the Mg2+ dependence for binding, and enhancement of integrin function by Mn2+ [36, 60, 116, 118]. The I domain contains a conserved cation binding site, the metal ion-dependent adhesion site (MIDAS) with clear preference for Mg2+/Mn2+. The MIDAS motif is critical for collagen recognition [69].

Structural and other studies of the α2 I domain have identified an inactive or closed conformation, an intermediate or low-affinity conformation, and an active or high-affinity conformation [3, 37, 66, 123, 136, 137, 140]. Experimental approaches have characterized the role that distinct I domain residues play in receptor conformation and ligand binding capability. Mutation of the Mg2+ binding site at T221 disrupts the MIDAS site and inactivates I domain function [112, 135]. Insertion of a disulfide bridge between helices locks the I domain into a high affinity conformation [124]. Within the α2 integrin I domain, amino acid E318 forms a salt bridge with amino acid R288, thereby maintaining the α2 integrin I domain in a closed conformation. Recent reports by Carafoli et al. indicate that mutation of E318 to alanine causes disruption of this salt bridge and promotes the transition to the open, high affinity conformation which enhances α2 integrin I domain binding to low-affinity ligands [19].

Crystal structures of the active α2 I domain E318W complexed with the GFOGER peptides revealed two domains bound to a single triple helix [19], suggesting that a single GxOGER motif in the heterotrimeric collagen V or the FACIT collagen IX, may support binding of the activated integrin. Similarly, a crystal structure of the analogous E317A mutant of α1 I domain also resulted in an opening of the helices [89], and modelling of a similar peptide, GLOGEN, onto E317A [25] allows similar conclusions to be drawn for $\alpha 1\beta 1$.

The $\alpha 2\beta 1$ integrin has high affinity for collagen Type I. Evaluation of the role of the $\alpha 2\beta 1$ integrin structure and function has led to the identification of a number of novel ligands. The other ligands can be subdivided into other

collagens, non-collagenous molecules with collagen-like triple helical structures, laminin and molecules with laminin domains, proteoglycans, as well as infectious organisms, primarily viruses, and other potential non-matrix ligands.

Among collagens, the α2β1 integrin preferentially binds fibrillar isoforms (I-III, V and XI). Integrin α2β1 also recognizes the network forming collagen IV [78], the beaded-filament forming collagen VI, and the transmembrane collagen XIII when in an active, high-affinity conformation [67]. Modulation of integrin conformation by cytoplasmic signals provides an integrin-specific mechanism for adjusting ligand affinity known as 'inside-out' signaling. However, the binding of purified recombinant α2 integrin I domain to collagen type I or IV reflects the same relative affinity for the ligand as does the parent integrin; indicating that differences in the integrin-binding motifs of these isoforms most likely account for the differential recognition by the integrin [18]. The development of overlapping sets of collagen-derived peptides, termed Toolkits, facilitated systematic mapping of motifs for integrin binding and identified the collagen sequence GFOGER as the major high-affinity binding motif for the α2β1 integrin [82, 83, 112]. The GFOGER motif, found in Type I, II and XI, is uniquely able to bind platelet integrin α2β1 without prior activation [124], suggesting the ability to induce the active conformation without the inside-out signals needed for lower-affinity motifs.

More recently, other collagens were defined as α2β1 integrin ligands. Collagen XVI, a member of the fibril-associated collagens with interrupted triple helices (FACITs), binds to the α2β1 integrin, as well as to the α1β1 integrin [33]. The α2β1 integrin ligand, collagen XXIII, a transmembrane collagen, has been reported as the primary apical binding partner for the integrin in keratinocyte adhesion in the epidermis [47, 53, 141].

Many molecules of the immune system contain segments of a collagen triple helix, including C1q. As discussed below, our laboratory showed that α2β1 integrin-mediated stimulation of an innate immune response required α2β1 integrin dependent-adhesion to C1q in an immune complex [34]. The full length α2β1 integrin and the α2 integrin I domain adhere to C1q as well as to members of the collectin family of proteins, including surfactant protein A and mannose binding lectin. The α2 integrin I domain adheres to C1q in the absence of activation. However, the activated E318A mutant of α2 I domain bound to C1q with higher affinity than wild type α2 integrin I domain.

As with collagens, adhesion to laminin isoforms is mediated by the α2 integrin I domain, however laminin binding only occurs in the active, high-affinity conformation [18, 22, 36]. Isolated full-length α2 integrin subunit has been shown to bind to laminin-111 (previously laminin-1) and laminin-332 (previously laminin-5). Netrin-4, a member of the netrin family of guidance signals, demonstrates high homology to the beta 1 chain of laminins and binds to the α2β1 integrin and to the α3β1 integrin [148]. To date, an extensive and detailed molecular analysis to identify the recognition site/s on laminin has not been performed. Laminin-binding has proven to occur constitutively in some cell types, and inducibly in others. However, the role of these adhesive events is not well understood.

Perlecan, a heparin sulfate proteoglycan, and its C-terminal fragment, endorepellin, bind the α2β1 integrin [45, 46]. The terminal globular domain of endorepellin, LG3, interacts directly with the α2 I domain. This interaction has been studied in the context of angiogenesis and shown to be important for α2β1 integrin-dependent angiogenesis.

Decorin, another small leucine-rich proteoglycan modulates α2β1 integrin matrix interactions by playing an important role in regulating extracellular matrix assembly as well as directly interacting with the integrin [13, 40, 52, 143]. Decorin binding to collagen has been shown to affect fibril formation by initially delaying lateral fibril growth and reducing average fibril diameter [142]. Additionally, decorin interacts with α2β1, but not α1β1 integrin, at a site distinct from the collagen-binding domain. Adhesive

interaction between decorin and the $\alpha2\beta1$ integrin was first identified in platelets, and later discovered to be important in angiogenesis.

Single nucleotide polymorphisms in the integrin $\alpha2$ gene, as discussed later in more detail, have an important role in the predisposition of patients to cardiovascular disease. One such minor allele difference (rs1801106; G1600A) has now been shown to attenuate adhesion of platelets to decorin but not to collagen and is associated with increased risk for recurrence of stroke [87]. The non-conservative amino acid substitution E534K, is the basis of the human platelet alloantigen system HPA-5, providing the first evidence of a functional effect of HPA-5 alleles.

The $\alpha2\beta1$ integrin serves as a receptor for many different infectious organisms. In many cases the organisms usurp $\alpha2\beta1$ integrin's routine biology for attachment, cell entry and transmission throughout the body. The best studied interaction of $\alpha2\beta1$ integrin is with echovirus (EV1) [10–12, 31]. EV1, is a human RNA virus which binds directly to the I domain of human $\alpha2\beta1$ integrin. Unlike most viruses that exploit integrin receptors, EV1 does not undergo clathrin-mediated endocytosis, but instead clusters on caveosomes and is internalized via a clathrin- and caveolin-independent macropinocytosis-like mechanism [73, 93]. Additionally, EV1 binding has been demonstrated to activate PKCα, while inhibition of PKCα signaling blocks EV1 internalization [138]. Interestingly, EV1, unlike other $\alpha2\beta1$ integrin ligands, preferentially binds the inactive, closed conformation of the integrin over the active, high affinity conformation [68].

Not only do infectious organisms utilize the integrin as a receptor, lectins that recognize high mannose glycans on viruses are produced from bacteria, algae, plants and animals and bind the $\alpha2\beta1$ integrin. A recently characterized anti-HIV lectin from Pseudomonas fluorescens Pf0-1 exhibited potent antiviral activity against influenza [121]. The lectin induced loss of cell adhesion and viral death that was dependent on binding to the $\alpha2\beta1$ integrin. Following lectin binding to the $\alpha2\beta1$ integrin, the complex was internalized to the perinuclear region and not recycled. The process resembled that described for echovirus mediated cell entry and death.

3.2 Signaling

The $\alpha2\beta1$ integrin plays a unique contribution in regulating cell migration, proliferation and survival. The $\alpha2$, but not the $\alpha1$, integrin cytoplasmic domain mediates p38 MAP kinase pathway activation and a migratory phenotype [80, 81]. Expression of the constitutively active small G protein Rac1 augmented p38 MAP kinase phosphorylation and migration in mammary epithelial cell expressing full length $\alpha2$ subunit. The role of the $\alpha2$-cytoplasmic domain in activation of the p38 MAP kinase pathway was also established in fibroblasts. Fibroblasts grown in three-dimensional collagen gels require the $\alpha2$-cytoplasmic domain for p38 MAP kinase activation that leads to $\alpha2\beta1$ integrin-mediated upregulation of collagen gene expression [62]. Together these results support an important and specific role for the $\alpha2$-cytoplasmic domain in mediating p38 MAP kinase activation. Similarly, the cytoplasmic domain of the $\alpha2$ integrin subunit specifically supports insulin-mediated S-phase entry [81]. The $\alpha2$, but not the $\alpha1$, cytoplasmic domain mediated activation of the cyclin E/cdk2 complex, which allows entry into S-phase in the absence of growth factors other than insulin. These results suggest that the $\alpha2$ integrin cytoplasmic domain and the insulin receptor synergize to regulate cell cycle progression.

More recently, Ivaska et al. suggested that the $\alpha2\beta1$ integrin induced protein serine/threonine phosphatase 2A (PP2A) activity in a collagen-specific manner [63]. In their studies, collagen-induced PP2A activation and resulting dephosphorylation of Akt and glycogen synthase kinase 3β (GSK3β) in Saos-2 cells was $\alpha2\beta1$ integrin-dependent. PP2A is a master regulator of a diverse set of cellular signaling pathways, so its interaction with $\alpha2\beta1$ integrin has the potential to dramatically increase the scope of the signaling activities of the integrin. Careful investigation of these putative signaling

mechanisms is necessary for a clearer understanding of the role for the integrin in various cell types.

3.3 The α2β1 Integrin: Expression and Function

In addition to differences in collagen recognition, expression of the integrin is dependent on cell type and stage of differentiation. The α2β1 integrin is primarily expressed in vivo by epithelial cells, platelets/megakaryocytes, and fibroblasts [146]. In addition, α2β1 integrin expression on T-cells and endothelial cells varies depending on differentiation and the state of activation [29, 55, 56, 144]. The roles and functions of the integrin are therefore highly dependent not only on cell type but on signals from other cells and the associated microenvironment.

The majority of earlier work defined the role and function of the α2β1 integrin by studies of human platelets and in vitro models. These early studies implicated the α2β1 integrin in a wide range of biologic and pathobiologic functions including platelet adhesion required for hemostasis and thrombosis, epithelial differentiation and branching morphogenesis, tumor biology, wound healing, angiogenesis, and inflammation and immunity. Much has been learned over the last 10 years since development of state of the art inhibitory antibodies and gene silencing approaches, novel in vitro culture systems, and new animal models including the global α2 integrin-subunit deficient and the more recent tissue-specific α2 integrin-subunit deficient mouse. These studies and their impact on our understanding of the integrin in human biology and disease will be reviewed.

3.4 Platelet α2β1 Integrin in Ligand Binding

Patient studies first established the link between α2β1 integrin and platelet function. In 1985 Nieuwenhuis identified a deficiency of platelet glycoprotein 1a (α2 subunit) in a patient with abnormal bleeding [106, 107]. Later other patients with either reduced levels of platelet expression of the α2β1 integrin or the presence of autoantibodies to the integrin were also described to exhibit impaired platelet activation by collagen but not by other agonists.

Studies using purified human platelets established the α2β1 integrin-dependent adhesion to collagens I-VIII in a Mg2+-dependent manner. Although the α2β1 integrin is expressed at relatively low copy number on platelets (2000–4000 copies per platelet), the integrin is required for firm attachment of platelets to collagen in the subendothelium after vascular injury [56, 85, 118]. Experiments with purified platelets from genetically modified α2-deficient mice confirmed these results. Platelets from α2-deficient animals fail to adhere to type I collagen under both static and flow conditions [24]. Platelets from animals heterozygous for the α2-null allele adhere to type I collagen to a lesser degree than platelets from wild type animals, consistent with a gene dosage effect.

Platelets however have not one, but two major collagen receptors: the high affinity α2β1 integrin and the lower affinity glycoprotein VI (GPVI)/Fc receptor γ-chain (FcRγ) complex [65, 102, 105]. Despite the significant evidence supporting the role of α2β1 integrin in platelet adhesion to collagen, the relative contribution and precise roles of α2β1 integrin and GPVI/FcRγ in collagen-induced platelet adhesion and activation is still a focus on experimental inquiry. The Santoro group originally proposed a two-step, two-site model of platelet adhesion and activation to collagen, in which the higher affinity α2β1 integrin supports the initial rapid platelet-collagen interaction that mediates platelet adhesion to vessel wall under conditions of flow [103, 116, 118, 128, 134]. This allowed the subsequent engagement of a lower affinity, signal-transducing co-receptor GPVI to bind collagen and mediate collagen-induced platelet activation and aggregation. GPVI, a member of the immunoglobulin superfamily noncovalently and constitutively associates with the FcRγ chain to form a multimeric signaling complex. In this model, the α2β1 integrin mediates strong

adhesion but does not contribute to platelet activation.

Other work raised question about the two-step, two-site model. Studies using a variety of agonists and inhibitors, defined the contributions and mechanisms leading to conformational changes resulting from integrin activation and provided evidence that the $\alpha 2\beta 1$ integrin can mediate GPVI-independent, collagen-induced platelet activation [59, 70, 75, 131]. Collagen-induced phosphorylation of PLC$\gamma 2$ and Syk was inhibited by antibodies that block $\alpha 2\beta 1$ integrin adhesion to collagen or by selective proteases that cleave the $\beta 1$ integrin subunit of the $\alpha 2\beta 1$ integrin. In other studies collagen-induced phosphorylation of c-Src was mediated by the $\alpha 2\beta 1$ integrin [61]. Platelet adhesion to intact collagen stimulated a different response than adhesion to GPVI-mimetics, further supporting distinct signaling from the $\alpha 2\beta 1$ integrin and GPVI/FcRγ [57, 70].

New work attempted to reconcile these conflicting stories. Auger et al. used flourescence video microscopy to monitor increases in intracellular free Ca2+ concentration ([Ca2+]i), an early stage in GPVI/FcRγ-mediated platelet activation, upon platelet adhesion to collagen under flow conditions [5]. In both human and mouse platelets under flow conditions, they identified a population of platelets that displayed an immediate increase in [Ca2 +]i upon collagen contact, as well as a second population of platelets that exhibited a delayed increase in [Ca2 +]i (1–30 s after adhering to collagen). The first population was unaffected by anti-$\alpha 2\beta 1$ integrin antibody blockade suggesting a GPVI/FcRγ-centric mechanism for both adhesion and activation as suggested by Nieswandt et al. The second population conformed to the traditional two-step model. The authors speculated that the apparently heterogeneous mechanism would allow for optimal response to different types of vascular injury. A similar study by Mazzucato et al. used inhibitory antibody-treated human platelets as well as mouse platelets from null animals to link short-lasting α-like and long-lasting γ-like [Ca2+]i oscillation peaks to $\alpha 2\beta 1$ integrin and GPVI signaling, respectively [97]. Interestingly, they found that $\alpha 2\beta 1$ integrin-mediated α-like calcium oscillations occur even in GPVI-null backgrounds indicating that inside-out priming of the integrin may also come from non-GPVI sources. Indeed Majoram et al. reported a role for platelet GPCRs, including protease activated receptor 1 and 4 (PAR1 and PAR4), in PLC-mediated $\alpha 2\beta 1$ integrin activation [94].

Together these studies demonstrated greater synergy between $\alpha 2\beta 1$ integrin and GPVI/FcRγ in mediating these processes than was previously understood. Resting platelets express the integrin in a low-affinity conformation. Activation, downstream of activation of GPVI, PAR1 or PAR4, or another pathway, leads to a conformational change to a high-affinity state which enhances adhesion to Type I collagen and promotes a more permissive binding to other ligands including Type IV collagen and laminin.

3.5 The $\alpha 2\beta 1$ Integrin: Genetic Risk for Hemostasis and Thrombosis and Much More

There is substantial variation in the baseline expression of $\alpha 2\beta 1$ integrin in the population; quantitative measurements of platelet surface membrane $\alpha 2\beta 1$ integrin expression indicate as much as a 10 fold difference among normal patients [64]. The mechanism of genetic regulation of the gene encoding the $\alpha 2$ integrin subunit has been best delineated. The variation is genetically determined and associated with three alleles of the $\alpha 2$ integrin subunit gene, *ITGA2* [84, 86]. The three alleles have been defined by 8 nucleotide polymorphisms in the coding region of *ITGA2* gene at nucleotide 807(C or T) and 873(G or A). Individuals carrying the 807T/873A allele express high levels of platelet $\alpha 2\beta 1$ integrin, whereas individuals carrying the 807C/873G allele exhibit low levels of $\alpha 2\beta 1$ integrin expression. Cheli et al. described another variant in CA repeat length in the *ITGA2* gene promoter that demonstrated linkage disequilibrium with variants in the coding region

[23]. Expression of α2β1 integrin may be similarly regulated in other cell types.

Genetic regulation of α2β1 integrin expression has meaningful biological implications, which have been most widely appreciated in the area of hemostasis and thrombosis. Kunicki et al. reported functional significance of α2β1 integrin expression levels by demonstrating that the number of α2β1 integrin molecules per platelet correlated with the ability of platelets to adhere to Type I collagen [85]. Clinical and epidemiologic studies based on genetic polymorphism analysis demonstrated direct clinical significance of allelic differences in levels of α2β1 integrin expression. The alleles associated with high levels of α2β1 integrin expression were associated with nonfatal myocardial infarction in individuals less than a mean age of 62 years, with an increased risk of developing diabetic retinopathy in patients with Type II diabetes mellitus, and with an increased risk of stroke [95, 119].

The original assumption was that increased integrin expression led to increased platelet adhesion to collagen and subsequent risk of thrombosis. Recently an alternative mechanism for the association was suggested. The level of α2β1 integrin expression correlated with mean platelet volume in humans and during megakaryocyte differentiation and proplatelet formation in mice [88, 126]. Surprisingly, platelet specific deletion of the integrin using the platelet factor 4 promoter-Cre construct and mice with a floxed ITGA2 gene demonstrated that mice lacking platelet-specific α2β1 integrin showed decreased megakaryocyte differentiation, diminished proplatelet formation and decreased mean platelet volume [49]. Since mice with global deletion of *ITGA2* failed to show altered megakaryocytic/platelet differentiation, compensation by alternative integrins, cell types, or pathways was sufficient to prevent this additional phenotype. Epidemiologic data linking levels of the α2β1 integrin expression with risk of pathologic thrombosis and other cardiovascular complications underscore the importance of further clarifying the role for α2β1 in platelet function.

3.6 The α2β1 Integrin During Wound Healing and Fibrosis

Early in vitro studies suggested that the α2β1 integrin was required for wound healing. Studies using skin explants ex vivo showed that keratinocyte-specific α2β1 integrin expression was re-oriented from the basal cell area to the forward-basal aspect of migrating keratinocytes where the integrin is in contact with type I collagen [114]. Keratinocyte migration into the wound was inhibited by antibodies against the α2β1 integrin [110].

In the late phase of wound healing after reepithelialization, tissue contraction of collagen fibers results in a strengthened scar. The scar is the result of extensive fibrosis, a process of tissue replacement by dense extracellular matrix composed of abundant collagen I. The α2β1 and the α1β1 integrins, both expressed by fibroblasts, are key regulators of collagen turnover in the skin, and other organs including the kidney [58, 62]. After binding to collagen, the α1β1 integrin activates a pathway that down-regulates collagen synthesis. In contrast, activation of the α2β1 integrin promotes collagen synthesis [99]. The alignment of the collagen fibers that occurs in healing wounds is recapitulated in three-dimensional collagen gels. The in vitro models provided evidence supporting critical roles for the α2β1 integrin wound healing and fibrosis.

Surprisingly, despite the results of in vitro and explant studies of wound healing, α2-deficient mice demonstrated no defect or delay in wound repair compared to wild-type animals [47, 152]. The morphology of the wounds also failed to demonstrate any difference in keratinocyte migration over exposed dermis at the wound site, suggesting that α2β1 integrin does not play an obligatory role in wound healing. No differences in scar formation or strength were noted.

Differences between the in vitro experiments and α2-null mouse model systems have several possible explanations. First, human and genetically altered mouse models may not be mechanistically equivalent. Acute loss-of-function as observed with use of inhibitory antibodies may

have different effects than the germ-line deletion of α2β1. In addition, antibodies that inhibit integrin binding may produce 'negative signaling' which is distinct from the absence of integrin signaling in the null context.

Interestingly, Zweers et al. and Grenache et al. both reported increased neoangiogenesis in the wound microenvironment of α2-null mice, providing in vivo evidence for an anti-angiogenic role for α2β1 integrin [47, 152]. The increased angiogenesis in the wound healing model was quite surprising. Many studies have focused on understanding the role of the integrin in vascular development and angiogenesis, as discussed below.

Fibrosis also occurs in other tissues; the involvement of α2β1 integrin is particularly well studied in the kidney [16]. Glomerulosclerosis, characterized by excessive collagen deposition in the glomerulus is the most common cause of end stage kidney disease. The specific role of α2β1 integrin in regulating glomerulosclerosis is somewhat controversial. Mesangial cells and podocytes express the α2β1 integrin. One report studying α2-null mice on the C57Bl/6 background suggested that the integrin protected from glomerular injury [44]. In contrast, a study in which α2-null mice were crossed with the COL4A3-null mice, a model of Alport disease demonstrated that α2β1 integrin expression exacerbates glomerular injury, decreased survival, and reduced glomerular matrix deposition and scarring [48].

Consistent with a role for the integrin in promoting collagen synthesis, Miller et al. showed that inhibition of integrin α2β1, using a high-affinity small-molecular weight inhibitor protects mice from glomerular injury [100]. The anti-α2β1 inhibitor also reduced collagen synthesis in wild type but not α2-null mesangial cells, consistent with the α2β1 integrin-dependence of its antifibrotic effect.

In contrast to the kidney, the α2β1 integrin appears to have an anti-fibrotic role in the lung. Xia et al. reported that in idiopathic pulmonary fibrosis (IPF), reduced fibroblast α2β1 integrin levels allowed escape from anti-proliferative signals that normally limit fibroproliferation after tissue injury [147]. Fibroblastic foci in IPF patients were shown to be characterized by low fibroblast α2β1 integrin expression. IPF fibroblasts demonstrated decreased α2β1 integrin-mediated PP2A phosphatase activity. Downstream increases in activity of GSK-3β and β catenin provided the proliferative signals that mark the pathological IPF fibroblast phenotype. Although this work provided an elegant model for how α2β1 integrin downregulation may contribute to the pathogenesis of IPF; the relevant mechanisms for α2β1 integrin loss remain uninvestigated. Additionally, it is unclear how the established role for α2β1 integrin in promoting collagen biosynthesis and ROS production may be involved. Are the disparate elements of α2β1 integrin function somehow context or tissue-specific? Reconciliation of the pro-fibrotic and anti-fibrotic properties of the α2β1 integrin demands further study in light of its potential clinical relevance.

3.7 The α2β1 Integrin and Angiogenesis/ Vasculogenesis

Angiogenesis is coordinated by a complex interplay between endothelial cells and their microenvironment. During VEGF-induced angiogenesis in vivo expression of α2β1 integrin is up-regulated and α2β1 integrin expression has been observed on the sprouting tips of neonatal blood vessels [38, 122]. Together these results suggested an important function for α2β1 in angiogenesis, however the precise nature of the integrin's role is still incompletely understood.

The earliest investigations into the functional role of α2β1 in angiogenesis employed inhibitory antibodies during in vitro studies. Early reports from Gamble et al. indicated that anti-α2β1 antibodies inhibited endothelial cell proliferation on collagen [41]. Soon after, Davis reported that anti-α2 inhibited lumen and tube formation by HUVECs in a 3D collagen matrix [28]. Later studies using planar type I collagen gel angiogenesis assays, confirmed that inhibition of α2β1 integrins with function blocking

antibodies disrupted tube formation [132]. Senger et al. demonstrated in vivo using subcutaneous matrigel plug angiogenesis assays in mice, that inhibition of α2β1 and α1β1 in combination decreased new vessel growth in the implanted plugs. Together these results suggested a pro-angiogenic function for the α2β1 integrin [122].

Studies from α2-deficient mice have yielded contradictory results. Several labs, including our own, reported not only normal developmental angiogenesis, but also increased neoangiogenesis during wound healing in genetically-altered α2β1 integrin-null mice [47, 149]. Similarly, our lab demonstrated that α2β1 integrin-deletion increased tumor angiogenesis in a growth factor-dependent manner via modulation of VEGFR-1 signaling. Additionally studies in the diet-induced obesity model also showed increased angiogenesis in α2-null mice compared to wild type mice [71]. The contradiction between the evidence for pro and anti-angiogenic functions for α2β1 integrin are not totally based of differences in mouse and human endothelial cells or in vivo compared to in vitro models. Cailleteau et al. used an α2 siRNA approach to alter integrin expression in HUVECs. These studies showed that α2β1 integrin engagement by laminin promoted endothelial cell cycle arrest and quiescence [17]. Additionally, α2β1 integrin binding to endorepellin in both human and mouse endothelial cells mediated the angiostatic effects [14, 46, 145].

Based on these inhibitory studies pharmacological inhibitors of α2β1 may have potential anti-angiogenic drug effects (see therapy section). Small molecule inhibitors (SMI) of α2β1 blocked both endothelial tube-formation in vitro and sprouting angiogenesis in zebrafish [115]. A more thorough understanding of the role for α2β1 in angiogenesis promises novel insight into clinical application of α2β1 integrin targeting compounds. Recent studies implicating the α2β1 integrin in notch signaling offer an alternative paradigm for understanding α2β1 integrin in angiogenesis [17, 39, 129]. The notch pathway coordinates sprouting angiogenesis by organizing endothelial cells into migratory 'tip' and proliferative 'stalk' cell conformations with differential capacity to respond to VEGF stimulation [54, 109]. Estrach et al. reported that α2β1-mediated laminin signaling is necessary but not sufficient for induction of the tip cell determinant, Dll4 [39]. Clarifying the functional relationship between α2β1 integrin and notch signaling in the endothelium is a promising avenue of future study.

3.8 The α2β1 Integrin in the Innate and Acquired Immune Response

The α2β1 integrin was initially identified as an integrin expressed at very late stages of T cell activation, thus the designation very late activation antigen-2 (VLA-2)(CD49b) [55, 56]. The α2β1 integrin was then noted on a variety of cells of the inflammatory and hematopoietic system, including activated T cells, but not naïve T cells in chronic inflammatory settings. Early studies showed that α2β1-dependent adhesion to collagen enhanced T cell receptor mediated T cell proliferation and cytokine secretion [120]. Boisvert et al. defined one possible mechanism; they reported that collagen I-stimulated, α2β1 integrin-mediated both activation-independent and T cell receptor-dependent interferon γ expression via the ERK and JNK MAPKs and PI3K/AKT signaling pathways [15].

The α2β1 integrin also influenced T cell activation by inhibiting fas ligand expression and apoptosis in effector T cells in a collagen I dependent manner [2, 42]. In animals, inhibitory monoclonal antibodies directed against the α2β1 integrin significantly inhibited the effector phase of both contact and delayed type hypersensitivity. These early results established a role for the α2β1 integrin in T cell mediated function. The role of the α2β1 integrin in the innate and acquired immune response has been an area of active investigation.

To better the define the role of the α2β1 integrin in T cell function, expression of the α2β1 integrin on T cell subsets and in response to antigenic challenges was investigated. Kassiotis et al. reported that expression of α2β1 integrin

defined two functionally distinct subsets of memory T cells that played a role in the response to infection and immunization [74]. α2β1 integrin expression was stably induced by antigen on approximately 50 % of memory T cells with helper function and stimulated production of tumor necrosis factor-α. The α2β1 integrin expressing, CD49b+, memory Th cells demonstrated enhanced ability to mediate macrophage activation and to kill of intracellular bacteria.

Sasaki et al. demonstrated that mature Th1 and Th2 cells exhibited distinct α2β1 integrin expression profiles [120]. Although naive Th cells did not express α2β1 integrin, Th1 cells acquired high levels of α2β1 integrin expression during maturation in an interferon-γ (IFN-γ) and interleukin (IL)-12-independent manner. This study suggested that high level α2β1 integrin expression on Th1, but not Th2, cells was functionally important, because stimulation of Th1 or Th2 cells with α2β1 integrin ligands caused selective activation of Th1 cells to produce interferon-γ after long-term culture.

Richter et al. studied α2β1 integrin expression during influenza infection in the lung [113]. During the acute phase of infection, the α2β1 integrin was expressed by a significant proportion of both CD4+ and CD8+ T cells in the lung; however, the integrin was expressed less frequently on memory cells, particularly CD8+ T cells. A similar expression pattern for the α2β1 integrin in the spleen was found in a model of lymphocytic choriomeningitis viral infection [1]. The data suggested that α2β1 integrin expression directed localization of CD4+ and CD8+ T cell subsets within the lung and promoted T cell migration within extralymphoid spaces, particularly during the acute phase of infection.

A role for α2β1 integrin expression by Th17 cells has been described. Boisvert et al. showed that human naïve CD4 T cells stimulated toward Th17 polarization preferentially upregulate α2β1 integrin [15]. Th17 cells adhered to collagens I and II, but not IVin an α2β1 integrin-dependent manner. α2β1 integrin-dependent adhesion combined with anti-CD3 antibody co-stimulated the production of IL-17A, IL-17F and IFN-γ by human Th17 cells.

The importance of α2β1 integrin to T cell memory has remained controversial. Work by several groups suggested that professional memory CD4 cells reside and rest in the bone marrow. Recently, Hanazawa et al demonstrated that memory CD4 cells expressed high levels of α2β1 integrin and that antibody-mediated inhibition of α2β1 integrin of memory CD4 cell precursors caused failure to transmigrate from blood through sinusoidal endothelial cells into the bone marrow [50]. These results suggested that the α2β1 integrin was required for the migration of memory CD4 cell precursors into their survival niches of the bone marrow.

In addition to its expression on activated T cells, the α2β1 integrin is expressed at high levels on almost all NK cells and mast cells, and on subpopulations of monocytes and neutrophils [4, 133]. Arase et al. identified the NK cell recognition epitope of the widely used DX5 pan-NK cell monoclonal antibody as CD49b or the α2β1 integrin. These investigators demonstrated that α2β1-expressing and nonexpressing subsets of NK cells are present in the mouse spleen and raised the possibility that α2β1 integrin expression is important in NK cell function. The role of the α2β1 integrin on subsets of neutrophils and monocytes has also been studied. One study found expression of the α2β1 integrin on extravasated neutrophils in human skin blister chambers and in the rat peritoneal cavity following chemotactic stimulation [144]. These studies, as well as others, suggested that the α2β1 integrin on neutrophils is involved in neutrophil migration from the vasculature into extravascular tissue in response to cytokine induction.

Work from our lab has clarified the function of the α2β1 integrin in mast call activation. We initially observed decreased inflammatory responses to Listeria monocytogenes in α2-null mice [34]. This innate immunity defect was determined to arise from a requirement for α2β1 integrin activation on peritoneal mast cells (PMCs) for mast-cell activation and cytokine release in vivo. We also identified C1q complement protein and collectin family members, including mannose binding lectin and surfactant protein A, as novel ligands for the integrin in

mast cell activation in vitro in response to Listeria. Since ligation of the α2β1 integrin alone was insufficient to activate cytokine secretion, we hypothesized that an additional signal emanating from a co-receptor was required to activate mast-cell cytokine secretion. We identified the required co-receptor as hepatocyte growth factor (HGF-R)/c-met [98]. We demonstrated that Listeria induced mast cell activation and cytokine secretion requires costimulatory signals from α2β1 integrin ligation to either type I collagen or C1q as well as c-met activation. The synergistic signal from the two coreceptors resulted in mast cell release of the proinflammatory cytokine IL-6 to trigger the early innate immune response.

3.9 α2β1 in Epithelial Biology

The α2β1 integrin is expressed at high levels on numerous epithelial cells including not only the squamous epithelium, but also ciliated columnar epithelium of the respiratory tract, the epithelial cells of the gastrointestinal tract and urinary tract, and the glandular epithelium of the breast [24]. In contrast to the high α2β1 integrin expression in the normal breast epithelium, markedly reduced or undetectable levels of α2β1 integrin were seen in poorly-differentiated carcinomas. Expression of α2β1-integrin was diminished or lost in a manner that correlated with a loss of epithelial differentiation and tumor progression in mammary carcinoma as well as other adenocarcinomas, including those of the prostate, lung, pancreas, and skin.

Our group's early studies focused on understanding the correlation between α2β1 integrin expression and a differentiated epithelial phenotype and conversely, whether dysregulated α2β1 integrin expression contributed to the malignant behavior of cancer cells. Gain of function and loss of function models in vitro suggested that α2β1 integrin expression contributed to the differentiated epithelial phenotype and branching morphogenesis of mammary and other epithelial cells [130, 150, 151]. These observations were supported by findings from other laboratories. Using a primary human nonmalignant, but immortalized, mammary epithelial cell line, Berdichevsky et al. and D'Souza et al. demonstrated that branching morphogenesis can be blocked by inhibitory monoclonal antibodies directed against the α2 integrin subunit or by altered α2β1-integrin expression mediated by the expression of the c-erbB2 proto-oncogene, respectively [9, 26, 27].

The development of genetically engineered mice with global deletion of *ITGA2* permitted further analysis of the role for α2β1 integrin in vivo. The major changes in branching morphogenesis in vitro were not fully recapitulated in vivo. The α2-null mice have only modest defects in mammary morphology. The in vitro experiments were designed to study a single integrin interaction on epithelial cells with only a small number of matrix molecules. Mammary gland in vivo consists of epithelial cells, fibroblasts, endothelial cells, and immune cells embedded in a complex matrix. The complexity in in vivo systems and compensatory mechanisms may both mitigate the consequences of α2β1 integrin-deficiency.

3.10 The α2β1 integrin Plays a Role in Cancer Progression

Interest in α2β1 integrin in breast cancer began with the observation of a strong correlation between diminished α2β1 integrin expression and a less differentiated phenotype. The α2β1 integrin–deficient mouse model provided our laboratory the opportunity to investigate a role for integrin in the development and progression of breast cancer in vivo. Our group demonstrated that in the spontaneous MMTV-neu mouse model of breast cancer, α2β1 integrin-deletion did not significantly alter the incidence of tumor development or tumor growth, but markedly increased hematogenous metastasis [111]. Increased metastasis in this model resulted in part from increased capacity for cancer cell intravasation.

Detailed in silico examination of publically available data from breast cancer patients supported this finding; expression of the α2 integrin

subunit, but not $\alpha 1$ or $\beta 1$ integrin subunits, was a prognostic indicator of decreased metastasis and better patient outcomes (Fig. 3.2). Similarly, retrospective analysis of lymph node-negative patients from the Wang cohort who relapsed with metastatic disease, revealed an inverse correlation between $\alpha 2\beta 1$ integrin expression and the occurrence of brain lesions; patients with greater than twice the average $\alpha 2\beta 1$ integrin expression suffered no brain metastasis whereas all nearly one third of all other patients suffered brain metastasis ($P = 0.0049$).

Expression of the $\alpha 2\beta 1$ integrin in prostate cancer was also predictive of metastasis and survival. The mouse and human studies supported the in vitro experimental analyses and the reported epidemiologic linkage between the single nucleotide polymorphisms regulating $\alpha 2\beta 1$ integrin expression and poor prognosis in patients with breast cancer [90]. Together these data suggested that $\alpha 2\beta 1$ integrin is a valuable biomarker for risk of metastasis in breast cancer.

Our data clearly showed in an animal model of breast cancer and human breast and prostate cancer that the integrin behaved as a metastasis suppressor. Data from other laboratories suggest that $\alpha 2\beta 1$ integrin's role in prostate and perhaps other cancers may be more complicated. In vitro, $\alpha 2\beta 1$ integrin was required but not sufficient for survival and metastasis of LNCaP prostate cancer cells to bone [91]. $\alpha 2\beta 1$ integrin protein and mRNA expression was enhanced in bone metastases to the level observed in normal, nonmalignant prostate tissue and significantly higher than primary prostate cancer lesions or metastasis to other sites such as lymph nodes [127]. Similarly, $\alpha 2\beta 1$ integrin expression accelerated experimental metastasis or tumor dissemination of melanoma and rhabdomyosarcoma or melanoma, gastric and colon cancer, respectively [7, 8, 51, 92, 96, 139].

Therefore, despite this progress several important questions remain concerning the role of the $\alpha 2\beta 1$ integrin in cancer biology. What is the precise molecular mechanism through which $\alpha 2\beta 1$ integrin loss enables increased intravasation? How does integrin down-regulation during breast cancer progression occur? Many other cancers including prostate, colon and lung cancer also appear to have $\alpha 2\beta 1$ integrin loss associated with cancer progression and metastasis. However, some cancers are associated with high $\alpha 2\beta 1$ integrin expression levels. Answers to each of these questions will provide novel insight into tumor biology, as well as suggesting new avenues for clinical application of the $\alpha 2\beta 1$ integrin as a biomarker or therapeutic target.

3.11 Therapies

Over the past several years there has been increased interest in pharmacological targeting of the $\alpha 2\beta 1$ integrin for treatment of thrombosis and angiogenesis [72]. The $\alpha 2\beta 1$ integrin is viewed as a safe target because although overexpression was associated with pathological clot formations, mice with integrin deletion lack severe bleeding defects, and inhibition causes only minimal increases in bleeding time. Compound 15, a nonpeptide inhibitor of the integrin, has been demonstrated to block platelet adhesion to collagen I under both static and flow conditions [16]. The inhibitor was originally designed to inhibit $\alpha 2\beta 1$ on platelets by locking the integrin $\alpha 2\beta 1$ in the inactive low-affinity conformation [100]. Additionally, in vivo, the compound inhibited thrombus formation in a mouse model and inhibited angiogenesis in a zebrafish model. Other $\alpha 2\beta 1$ inhibitors have shown similar effects; BTT-3016, a sulfonamide derivative prevented platelet aggregation and reduced thrombus formation in a vascular injury model [108]. Another sulfonamide derivative that targets $\alpha 2\beta 1$, E7820, is currently in phase II clinical trials as an adjuvant therapy for metastatic colon cancer [77, 101]. The clinical impact of pharmacological targeting the $\alpha 2\beta 1$ integrin will require further time and experimentation.

3.12 Summary and New Directions

It is increasingly clear that the $\alpha 2\beta 1$ integrin plays a nuanced but important role in critical cell functions in many different cell types. Several

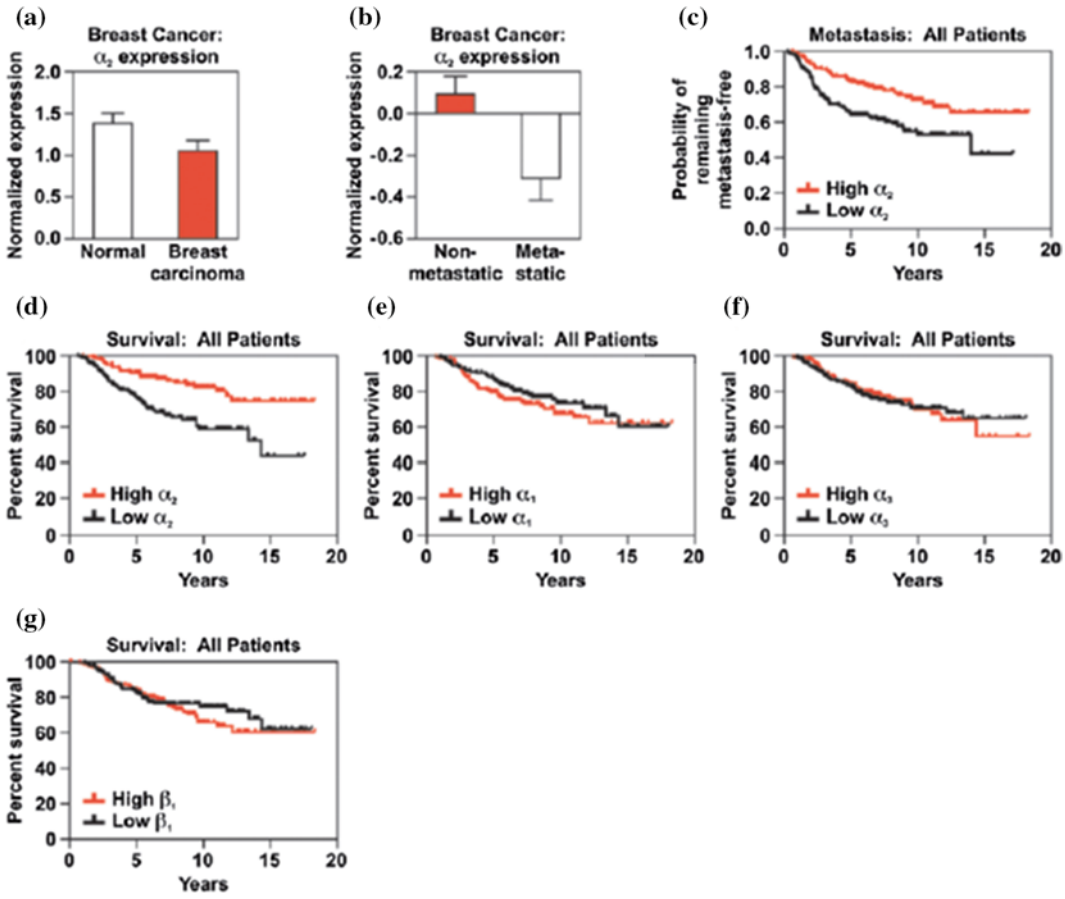

Fig. 3.2 Decreased α2β1 integrin mRNA expression predicts metastasis and decreased survival in breast cancer patients. **a** Expression of the α2 integrin was significantly decreased in breast carcinomas (n = 40) compared with normal breast tissue (n = 7) (P = 0.038). (**b–g**) Analysis of the NKI-295 cohort correlates expression of α2 integrin, but not other integrins, with metastasis (**b** and **c**) and patient survival (**d–g**). The α2 integrin expression was substantially reduced in patients with metastases (n = 101) when compared with non-metastatic patients (n = 194, P = 0.0038) (**b**). Log-rank analysis demonstrates that high-level α2 integrin expression correlates with the probability of both remaining metastasis-free (**c**, P = 0.0022) and with improved long-term survival (**d**, P < 0.0001). In contrast, expression of the α1 (**e**, P = 0.2639), α3 (**f**, P = 0.9509), and β1 (**g**, P = 0.5) integrin subunits did not correlate with patient survival (Reprinted from Fig. 6, [111])

new studies have suggested previously undocumented roles for the integrin in diseases ranging from type 2 diabetes, to dwarfism. In platelets, the combination of animal and in vitro studies have slowly revealed a more nuanced yet equally important role for the integrin than had previously been imagined. The recent development of tissue-specific α2-null mice promises to bring similar clarity and complexity to our understanding of α2β1 integrin function in inflammation, angiogenesis and tumor biology in the years ahead.

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Integrin $\alpha 10\beta 1$: A Collagen Receptor Critical in Skeletal Development

4

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Abstract

Integrin $\alpha 10\beta 1$ is the most abundant collagen-binding integrin in cartilaginous tissues and its expression pattern is distinct from that of other collagen-binding integrins. In vitro and in vivo studies have identified integrin $\alpha 10\beta 1$ as a unique phenotypic marker for chondrocyte differentiation and a crucial mediator of cell-matrix interactions required for proper cartilage development. This chapter describes the structure of the integrin subunit $\alpha 10$, the tissue distribution of the integrin $10\beta 1$ and updates available information regarding its regulation and ligand binding. We also summarize current information on the functional roles of $\alpha 10\beta 1$ in chondrogenesis of mesenchymal stem cells and in skeletal growth.

Keywords

Integrin $\alpha 10\beta 1$ · Collagen · Chondrocyte · Mesenchymal stem cell · Chondrodysplasia

4.1 Introduction

The integrin $\alpha 10\beta 1$ was identified as a collagen type II binding receptor on chondrocytes in 1998 by Camper et al. [9]. Earlier studies in our laboratory had indicated that an unknown α subunit in the $\beta 1$ integrin family with a molecular weight of approximately 160 kDa was present on chondrocytes and chondrosarcoma cells [16]. To identify this integrin subunit, large quantities of chondrocytes (2.5 billion cells) were collected from bovine articular cartilage and integrin $\alpha 10$ was isolated by affinity purification of the chondrocyte lysate on a collagen II-Sepharose column. The human ortholog of the $\alpha 10$ subunit was subsequently characterized using a human chondrocyte library [9]. With an antibody raised against the cytoplasmic domain of $\alpha 10$ we could show that integrin $\alpha 10\beta 1$ is a major collagen-binding integrin on chondrocytes and that it is highly expressed in cartilage, both during development and in adult tissues [9, 10]. We have also

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shown that fibroblast growth factor -2 (FGF-2) upregulates expression of α10 and improves chondrogenic potential of mesenchymal stem cells (MSCs) [30]. In 2005 we demonstrated that mice lacking the integrin α10β1 have defects in the cartilaginous growth plate and, as a consequence, develop growth retardation of the long bones [4]. A recent study revealed that a naturally occurring mutation in the canine α10 integrin gene is responsible for chondrodysplasia in hunting dog breeds [19], supporting a critical role for α10β1 in skeletal development.

This chapter summarizes current knowledge on structure, distribution and function of the integrin α10β1.

4.2 The α10 Structure

The α10 subunit has a M_r of around 160 kDa under reducing conditions as determined by SDS-PAGE. When compared to the other collagen-binding integrins on cultured chondrocytes, the integrin subunit α10 appears distinctly smaller than α1 in SDS-PAGE, slightly smaller than α11 and similar in size to the α2 subunit under reducing conditions [9].

Sequence analysis of the four collagen-binding integrins shows that α10 displays 43 % sequence identity to α11, 33 % to α1 and 31 % identity to α2 at the amino acid level. Similar to the other collagen-binding integrin subunits α1, α2 and α11, the I-domain in α10 is encoded by exons 6-9. At the protein level, α10 I domain extends from C^{140}-G^{337} and consists of 198 amino acids which is inserted in the *N*-terminal region of the extracellular domain between cation binding sites two and three. Three cysteine residues are present within the α10 I-domain, compared to one cysteine in α11, two in α1 and three in α2. Like the other I-domain-containing collagen-binding integrins, the α10 I-domain contains a MIDAS (metal ion-dependent adhesion site) motif [9]. The overall identity between α10 I-domain and the I-domains of the other collagen-binding integrins is high with the highest identity to the α11 I-domain (60 %) [14].

The conserved sequence in the transmembrane/cytoplasmic region of α10 is GFFAH and not GFFR/KR as in most other integrins [9]. Analysis by an in vitro glycosylation method has shown that the length of the α10 transmembrane (TM) domain is 29 amino acids and extends to the Ala in the sequence GFFAH, 1-2 amino acids further at the C terminus compared with the TM domain of other α subunits [26]. Thus, the cytoplasmic domain of α10 consists of 16 amino acids.

4.3 The α10 Gene

The human α10 gene, (*ITGA10*), which consists of 30 translated exons distributed over a region of 19kb has been located to chromosome 1, locus q21 [5, 20]. It is the only integrin located on this chromosome [5].

The mouse α10 gene (*Itga10*), located on chromosome 3, F2.2, has a homology of 90 % with *ITGA10*. The highest homology is found in the I-domain (97 %) [5]. *Itga10* consists of 30 translated exons spanning a region of about 18 kb genomic DNA. Primer extension analysis determined that a major transcription start site is located 38 nucleotides (nt) upstream from the translation initiation site ATG. The 5′-flanking region of the transcription site at –38 nt lacks a TATA box, as is the case for most other integrin subunits [5].

We have previously demonstrated that the human α10 subunit exists as two splice variants due to alternative splicing of exon 25 (114 nt). The spliced domain is located extracellularly, close to the transmembrane region. Both forms are expressed at the mRNA level in human chondrocytes but it is not clear if the smaller form, lacking exon 25, is present on the cell surface of chondrocytes [5].

In contrast to human *ITGA10*, mouse exon 26 and not exon 25 is alternatively spliced [5]. In the spliced variant, exon 26 containing 144 nt is extended into the intron by 62 nt and results in a shift in the reading frame and a premature stop codon [5].

Extracellular splice variants have been reported for other integrin α-subunits, although the functions of the variants are not understood [11]. Similar to α10, α7 and αIIb have alternatively spliced extracellular regions close to the transmembrane spanning domain [7, 21]. In addition, α11 has an inserted region of 22 amino acids in exon 20 [32].

Sequence analysis of a promotor region of human *ITGA10*, together with functionality tests, have identified the transcription factors AP-2ε and Ets-1 as regulators of the integrin α10 gene in chondrocytes [34]. These transcription factors are also known to control expression of integrin α10 in melanoma cells [33].

Interestingly, a truncating mutation of the *ITGA10* gene on canine choromosome 17 was recently shown to cause chondrodysplasia, short stature dwarfism, in dogs (see below) [19].

4.4 Distribution of Integrin α10β1

Expression analysis of mouse and human tissues has shown that distribution of integrin α10β1 is quite restricted [10, 14]. It is strongly expressed in the cartilage of joints and in other cartilage-containing tissues such as vertebrae, ribs, trachea and bronchi [10]. Integrin α10β1 is found where collagen type II is expressed and is an excellent cellular marker of cartilage tissue. Integrin α10β1 is also expressed on chondrocytes in the growth plate and on bone lining cells in the trabecular bone (Fig. 4.1).

We have also found α10β1 on cells in some fibrous tissues such as perichondrium, periosteum and endosteum, and in the fascia lining skeletal muscle fibers [10, 30]. These tissues are known to house mesenchymal progenitor cells with the potential to develop into different cell types [3, 15, 22]. In addition, integrin α10 is present in the junctions between cartilage/bone and ligaments [10].

In the vertebral column, α10 was detected by immunohistochemistry in the cartilage of the vertebral body and in the inner annulus fibrosus of the intervertebral discs [10]. In both tissues, α10 expression was co-localized with collagen II. The outer annulus fibrosus, which contains collagen I, was negative for α10. Interestingly, α11 has been detected in the outer annulus fibrosus [28].

An mRNA array of human tissues has also suggested expression of α10 outside of the musculoskeletal system, e.g. in heart and aorta [14]. When we analyzed α10 protein in the mouse heart, we found immunodetectable expression in the aortic and atrioventricular valves but not in the heart muscle [10]. Interestingly, collagen II is expressed in the heart valves during early development but the role for this collagen during heart development is not known [27]. On the other hand, we have not been able to confirm expression of α10 on the protein level in aorta. However, we have detected α10 in atherosclerotic plaque both in human and in a mouse model (Lundgren-Åkerlund and Hultgård-Nilsson, unpublished results). The plaques may represent the strong aorta signal in the mRNA tissue array [14]. In unpublished studies we found that α10 colocalized with collagen II in atherosclerotic plaques.

The expression of integrin α10β1 in tissues that are exposed to high mechanical load such as articular cartilage, vertebral column and heart valves implicates a role for α10β1 integrin in mechanical integrity and/or in mechanical signaling of these tissues.

We have earlier published that malignant melanoma express integrin α10 and that antibodies blocking α10 reduce migration of the melanoma cells in vitro [33]. It is not known if α10 is present in other tumors.

4.5 Integrin α10β1 in Mouse Limb Development

The major part of the mammalian skeleton is laid down by a process called endochondral bone formation. Cartilage moulds form during embryogenesis via the sequential steps of mesenchymal cell condensation, chondroprogenitor commitment and chondrocyte differentiation under the control of multiple mechanisms, including cell-cell and cell-matrix interactions,

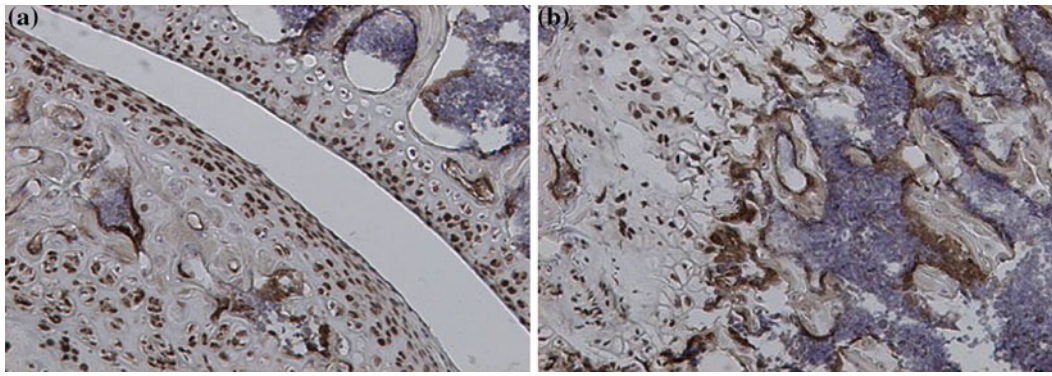


Fig. 4.1 Immunohistochemical localization of the integrin $\alpha 10\beta 1$. Mouse hind limbs from 8-week-old mice were cryosectioned and stained with an affinity purified polyclonal antibody recognizing the cytoplasmic domain of $\alpha 10$. The integrin $\alpha 10\beta 1$ was expressed by all chondrocytes in the epiphyseal cartilage and in the growth plate and also by cells in the trabecular bone

cellular signaling and transcriptional and translational regulation. Most fetal cartilages are transient and will gradually be replaced by trabecular bone during a series of events including chondrocyte proliferation, hypertrophy and apoptosis followed by cartilage matrix mineralization, vascularization and matrix degradation. These events take place within the growth plate. Proliferation, matrix production and hypertrophy of chondrocytes in the growth plate are essential to achieve longitudinal elongation of endochondral bones. Oppositely, in articular cartilage, the chondrocytes acquire a stable phenotype that resists hypertrophy and vascular invasion, thus maintain a mechanically adequate ECM throughout the life [1].

We have demonstrated that $\alpha 10\beta 1$ is the major collagen-binding integrin in the cartilage during development of the skeleton in mouse [10]. Expression analysis of the hind limb revealed that $\alpha 10$ appears at embryonic day 11.5 (E11.5) at the onset of chondrogenesis as determined by collagen type II expression. At E13.5, $\alpha 10$ is present throughout the anlage as well as in the perichondrium and in the mesenchyme just outside of the perichondrium. In newborn mice, $\alpha 10\beta 1$ is expressed by all chondrocytes in the growing epiphyseal and growth plate cartilage. 4 weeks after birth, $\alpha 10$ is prominent both at the articular surface and in the growth plate [10]. $\alpha 10$ is also detected in the inner cartilaginous region of the meniscus where collagen type II is expressed.

Interestingly, $\alpha 10$ is expressed by cells in the ossification groove of Ranvier [10]. The ossification groove contains precursors for both chondrocytes and osteoblasts and has been suggested to be involved in growth of the bone [23, 24].

4.6 Ligands Specificity

The integrin subunit $\alpha 10$ was originally isolated by affinity purification on a collagen type II-column [9]. Experiments using $\alpha 10\beta 1$-expressing C2C12 cells and an $\alpha 10$ blocking monoclonal antibody have confirmed that $\alpha 10\beta 1$ is a receptor for fibril-forming collagen types II and XI and for the FACIT (fibril-associated collagen with interruptions in triple helix) collagen type IX in vitro (Fig. 4.2). The $\alpha 10$-expressing cells also bound to the beaded filament-forming collagen VI but, in contrast to the other collagens, we could not block the adhesion of $\alpha 10$ expressing C2C12 cells to collagen VI with the monoclonal antibody. This might be explained by a different binding mechanism of $\alpha 10\beta 1$ to collagen VI or, alternatively, that other collagen interacting receptors are involved in the attachment of C2C12 cells to collagen VI. We have also found that collagen type X, expressed by

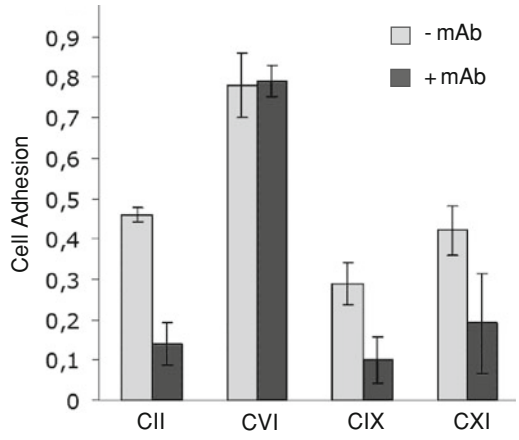


Fig. 4.2 Adhesion of α10β1 expressing cells to the cartilage collagen types II, VI, IX and XI. C2C12 cells, expressing α10β1 as the only collagen-binding integrin, were allowed to adhere for 1 h to collagen-coated (10 μg/ml) culture dishes in the absence (−mAb) or in the presence (+mAb) of a blocking monoclonal antibody directed to the I-domain of α10 (5 μg/ml). The numbers of adhered cells were compared to the total number of cells added to the wells (1 = 100 %). The numbers represent the mean ± S.D. of triplicate experiments. The antibody reduced adhesion of α10β1 cells co collagen II, IX and XI but not to collagen VI

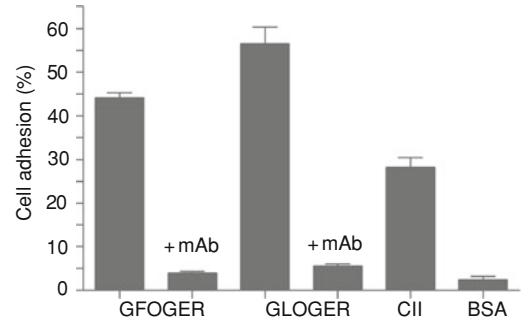


Fig. 4.3 Adhesion of α10β1 expressing cells to the collagen peptides GFOGER and GLOGER. C2C12 cells, expressing α10β1 as the only collagen-binding integrin, were allowed to adhere for 1 h to culture dishes coated with 10 μg/ml of the collagen peptides GFOGER and GLOGER and collagen type II (CII) and blocked with 1 % bovine serum albumin (BSA). The adhesion experiments were performed in the absence or in the presence (+mAb) of an α10 blocking monoclonal antibody (5 μg/ml). The number of adhered cells is shown as a percentage of the total number of cells added to the wells. The numbers represent the mean ± S.D. of triplicate experiments. From Bryngelson Ohlsson [8]

hypertrophic chondrocytes, can mediate adhesion of α10β1-expressing cells (Lundgren-Åkerlund, unpublished results).

Our findings that α10β1 is present in non-cartilaginous tissues implicates that α10β1 in vivo interacts with other ligands than cartilage collagens. Indeed, we have found that α10β1-expressing cells also interact with collagen types I and IV (Lundgren-Åkerlund, unpublished results).

Using recombinant I-domains, the study by Tulla et al. [29] suggested that the α10 I-domain, similar to the α1 I-domain, has a preferred affinity for collagen IV (a basement membrane collagen) and collagen VI over the fibrillar collagen types I-III.

It has previously been reported that I domains of the collagen-binding integrin subunits α1, α2 and α11 interact with the trippel helical collagen I peptides GFOGER and GLOGER [25, 35, 37]. We have found that α10β1-expressing cells adhere to the peptides GFOGER and GLOGER and that the adhesion can be inhibited by an α10-blocking monoclonal antibody recognizing the I-domain of α10 (Fig. 4.3) [8]. Recently, Käpylä et al. has demonstrated that all collagen integrin receptors, including α10β1, bind collagen IX via a novel, GFOGER-independent mechanism which does not resemble interactions with other collagen types [17].

4.7 Integrin α10β1: A Chondrogenic Differentiation and Potency Marker

Primary chondrocytes, with a differentiated phenotype, express α10β1 on the cell surface and synthesize the cartilage specific molecules, collagen type II and aggrecan. During monolayer cultures chondrocytes are known to dedifferentiate as characterized by a decrease in expression of collagen II and increased synthesis of fibrous matrix molecules, such as collagen type I [6]. We have found that expression of integrin α10β1 is gradually downregulated from the cell surface during dedifferentiation of chondrocytes in monolayer cultures (Fig. 4.4).

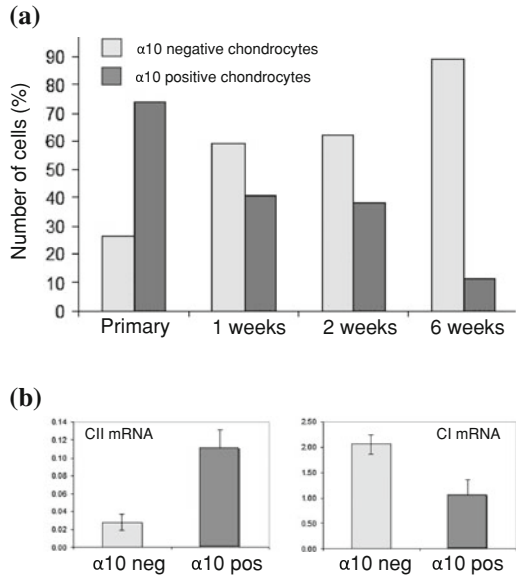


Fig. 4.4 Expression of α10β1 and collagen types I and II in cultured chondrocytes. **a** Chondrocytes were isolated from human articular cartilage and the expression of integrin α10β1 was analyzed on primary chondrocytes and on chondrocytes cultured in monolayer for 1, 2 or 6 weeks by flow cytometry. The cells were passaged once a week. The bars show percentage of α10 negative and α10 positive chondrocytes at each time point. Expression of α10 gradually decreased with time in culture and was approximately 10% after 6 weeks. **b** After 6 weeks of culture, mRNA levels of collagens I and II were analyzed in α10 positive and α10 negative sorted chondrocytes. Expression of collagen II was higher on α10 positive chondrocytes while expression of collagen type I was higher on α10 negative chondrocytes

After 6 weeks of culture, only about 10% of the cells expressed α10. However, after FACS sorting, we found that the α10 positive cells had a higher collagen II/collagen I mRNA ratio compared to the α10 negative cells. Furthermore, dedifferentiated chondrocytes lacking α10 were able to restore expression of integrin α10β1 and redifferentiate when the cells were transferred to three-dimensional alginate cultures (Fig. 4.5). This observation implicates that integrin α10β1 is a unique cellular marker for the differentiation state of the chondrocytes. In agreement, Gouttenoire et al. [13] showed that α10 together with the chondrogenic collagen type IIB isoform are expressed by differentiated mouse chondrocytes after treatment with BMP2, while TGFβ1 stimulated the expression of the non-chondrogenic procollagen type IIA and α11 integrin. In another study it was demonstrated that aggregation of phenotypically stable chondrocytes is mediated by integrin α10β1 and collagen type II interaction [12].

We have previously reported that integrin α10β1 is present on human mesenchymal stem cells (MSCs) and that its expression increases during *in vitro* chondrogenesis in aggregate cultures [30]. We have also reported that extended monolayer culturing of MSCs downregulates integrin α10, while treatment of the cultured MSCs with fibroblast growth factor-2 (FGF-2) increases expression of α10. In contrast, FGF-2 treatment of the MSCs decreases expression of α11 [30] (Fig. 4.6). Transforming growth factor-β3 (TGF-β3), on the other hand, was found to decrease expression of α10 and increase expression of α11 on MSCs [30]. The effects of FGF-2 and TGF-β3 on α10 and α11 expression observed in MSCs appears to extend to cultured human and bovine chondrocytes (Lundgren-Åkerlund and Aszòdi, unpublished results).

We have also reported that FGF-2-induced upregulation of α10 in MSCs enhances chondrogenesis and synthesis of cartilage molecules such collagen type II and aggrecan in pellet cultures [30]. This demonstrates that α10β1 is a unique cell surface biomarker and potency marker of MSCs with chondrogenic potential and will serve as a valuable tool in the quality assurance of chondrocytes and chondrogenic MSCs used in cartilage repair.

4.8 Loss of Integrin α10β1 Leads to Chondrodysplasia

The essential role of integrin-mediated attachment and signaling in endochondral bone formation was first demonstrated by conditional inactivation of the floxed β1 integrin gene in the entire cartilaginous skeleton using a transgene which drives the expression of the cre recombinase under the control of the collagen II

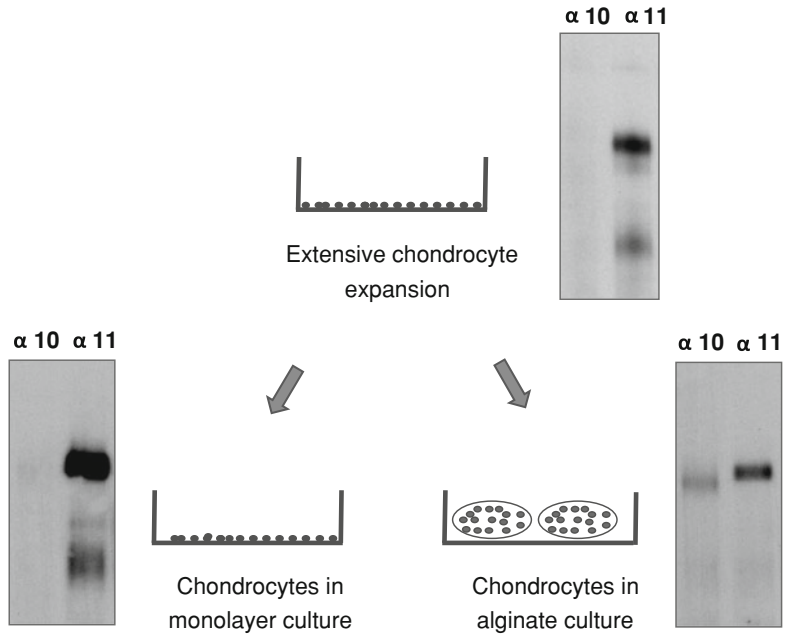


Fig. 4.5 Integrin α10β1 expression is restored when dedifferentiated chondrocytes are transferred to three-dimensional alginate culture. Human chondrocytes were dedifferentiated in monolayer cultures until expression of the α10 protein was lost (8 weeks of culture, five passages, in this experiment). One part of the chondrocytes was then transferred to alginate beads for redifferentiation in three-dimensional culture. The other part was kept in monolayer culture. The chondrocytes were cultured for additional 2 weeks and then cell surface biotinylated, lysed and immunoprecipitated with antibodies directed to α10 and α11 followed by separation by SDS-PAGE and western blot analysis. The results demonstrate that expression of α10 was restored in chondrocytes in alginate culture while expression of α11 appeared to decrease in alginate compared to monolayer cultures. This demonstrates that integrin α10β1 is a cellular marker for staging the differentiation status of chondrocytes

promoter (*Col2a1cre*) [2]. We have shown that mutant mice ($\beta1^{fl/fl}$-*Col2a1cre*$^{+}$) develop perinatal lethal chondrodysplasia, characterized by the lack of columnar growth plate, reduced chondrocyte proliferation, abnormal cell shape and distorted collagen fibrillar network in the ECM. These observations have demonstrated that β1 integrin-mediated cell-matrix interactions are mandatory for chondrocyte geometry, motility and cytokinesis, essential mechanisms necessary for the proper formation and function of the growth plate.

Among mice lacking an α integrin subunit, only the knockout of the α10 integrin gene [4] results in skeletal abnormalities which partially recapitulate the phenotype of the $\beta1^{fl/fl}$-*Col2a1cre*$^{+}$ mice (Fig. 4.7). We have shown that α10-deficiency is dispensable for life but causes chondrocyte shape change and mild disorganization of columnar arrangement resulting in moderate growth retardation. Some other abnormalities such as the shorter hypertrophic zone, increased apoptosis and reduced chondrocyte proliferation also contribute for growth plate dysfunction in both mouse models. It is particularly interesting that the chondrocyte cell cycle apparently is modulated by α10β1 integrin. Both α10- and β1-deficient chondrocytes display delayed G1/S transition accompanied by increased nuclear translocation of Stat1 and Stat5a, two members of the family of signal transducers and activators of transcription, inducing the upregulation of cell cycle inhibitors p16 and/or p21 which in turn decreases the proliferation rate [2, 4]. Another striking phenotype present in the two knockout strains is the

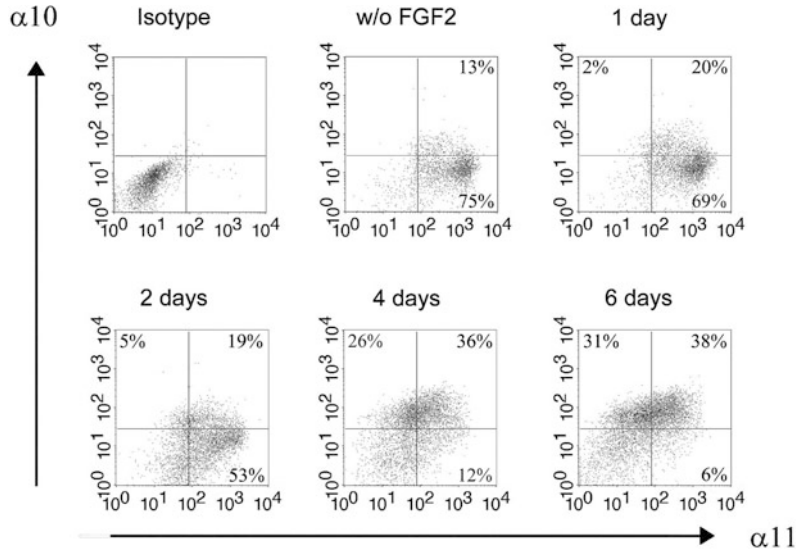


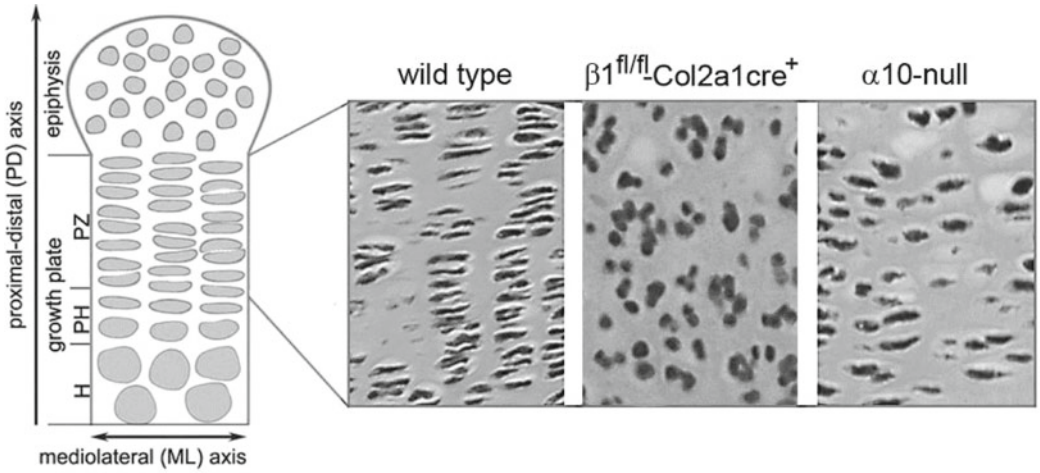
Fig. 4.6 FGF-2 increase expression of α10 and decrease expression of α11 on mesenchymal stem cells. Human bone marrow derived mesenchymal stem cells (MSCs) were isolated by plastic adherence and cultured for 4 weeks. The MSCs were then transferred to 6-well dishes and stimulated with 10 ng/ml of fibroblast growth factor-2 (FGF-2,) for 1, 2, 4 and 6 days and subsequently analyzed by flow cytometry using antibodies directed to α10 and α11. The *upper left panel* represent the background staining using an isotype antibody. The results show that treatment with FGF-2 gradually increases expression of α10 (y-axis) and decreases expression of α11 (x-axis). From Varas et al. [30]

reduced density of the collagen matrix implying a role of integrin α10β1 for matrix assembly. Since the β1-deficient chondrocytes express normal levels of collagen II, this matrix defect suggest the direct involvement of β1 integrins in collagen fibril polymerization and/or incorporation of the fibrils into the collagen meshwork.

Despite the aforementioned similarities, the skeletal phenotype is more severe in *β1 $^{fl/fl}$-Col2a1cre^{+}* mice and some abnormalities such as the defective cytokinesis or the disrupted actin network are only observed in β1 null chondrocytes suggesting partial redundancy among β1 integrins. In vitro assays have indeed demonstrated comparable adhesion and spreading of primary wild type and α10 null chondrocytes on fibrillar collagens [4]. The integrin α1β1, which is expressed on chondrocytes in the articular cartilage, is a strong candidate for compensating the α10-deficiency. The integrins α2β1 and α11β1, on the other hand, appeared to be absent or only weakly expressed in the cartilage and on isolated chondrocytes [4, 10]. However, mice lacking α1 integrin show no growth plate phenotype but develop early onset osteoarthritis [36], an ageing-dependent degeneration of the articular cartilage. We have shown in 4-week-old mice that in contrast to the epiphyseal cartilage, α1 is only weakly expressed in the growth plate [10]. This may explain why mice lacking α1 integrin have no growth plate abnormalities. Integrin α10, on the other hand, was strongly expressed both in the epiphyseal cartilage and in the growth plate. Taken together, the studies with genetically modified mice support a hypothesis for both overlapping and distinct role of collagen-binding integrins in skeletal development and function where α10β1 may play a specific role in growth plate morphogenesis and skeletal growth.

Inherited chondrodysplasias are caused by mutations in a range of gene families encoding e.g. ECM proteins, transcription factors, growth factor receptors, enzymes, or signaling molecules, but so far no integrin mutations have been associated with these skeletal disorders in

(a)



(b)

phenotype	β1 fl/fl - Col2a1cre +	α10-null
chondrodysplasia	severe, lethal	mild, vital
shape of proliferative chondrocytes	round	moderatelly rounded
growth plate disorganization	complete lack of columns	moderate disorganization
hypertrophic zone	reduced	reduced
chondrocyte proliferation	decreased	decreased
apoptosis	increased	increased
cytokinesis defect	severe	none
adhesion to collagen II	diminished	normal
actin cytoskeleton	abnormal	normal
collagen network	reduced density	slightly reduced density
collagen fibril diameter	thickened	thickened

Fig. 4.7 The lack of α10β1 integrin results in moderate chondrodysplasia and growth plate dysfunction. **a** In normal growth plate, chondrocytes form horizontal zones reflecting their differentiation stage (proliferative, pre-hypertrophic and hypertrophic). The flattened proliferative cells are oriented with their long axes perpendicular to the direction of the longitudinal growth and arranged into vertical columns. Proliferative chondrocytes are gradually rounding up and lose their columnar organization in mice lacking α10β1 integrin *(α10-null)* or all β1 integrin-containing heterodimers ($\beta1^{fl/fl}Col2a1cre^{+}$) on chondrocytes. **b** Comparison of the cartilage phenotype in *α10-null* and $\beta1^{fl/fl}Col2a1cre^{+}$mice

human [18, 31]. Recently, genetic analysis of two dog breeds has revealed that a naturally occurring mutation in the α10 integrin gene is responsible for canine chondrodysplasia [19]. The Nordic hunting dogs Norwegian Elkhound and Karelian Bear Dog display disproportionate short-limbed dwarfism characterized by growth plate abnormalities resembling the α10 knockout mouse phenotype. Using a genome-wide approach, a recessive mutation in *ITGA10* was shown to be segregated with the disease in both breeds. The nonsense mutation p.Arg695* in

exon 16 was predicted to produce a truncated protein lacking the cytosolic tail, the transmembrane domain and part of the extracellular domain. As judged by western blot, the truncated protein was undetectable in tracheal cartilage suggesting a loss of function of $\alpha 10\beta 1$ [19] in these dogs.

The current nosology and classification of human genetic skeletal disorders show that the causative gene for many human genetic skeletal disorders are still unknown [31]. Our results from the $\alpha 10$ knockout mouse model together with the results from the natural mutation in the canine ITGA10, showing that loss of function of the integrin $\alpha 10\beta 1$ gene leads to disproportionate chondrodysplasia, suggest that ITGA10 is a likely candidate gene responsible also for human disproportionate chondrodysplasias.

4.9 Perspectives

Integrin $\alpha 10\beta 1$ has a specific role during skeletal development that does not overlap with other collagen-binding integrins. However, very little is known about the molecular mechanisms behind the specific function of $\alpha 10$ in skeletal development and its involvement in different pathological conditions such as chondrodysplasias and osteoarthritis. The fact that integrin $\alpha 10$ is expressed in tissues that are exposed to high mechanical load implicates a role for $\alpha 10$ in mechanical integrity and/or in mechanical signaling of these tissues.

Integrin $\alpha 10\beta 1$ is a prominent collagen receptor on chondrocytes and its expression correlates with expression of cartilage matrix molecules such as collagen type II. This makes $\alpha 10$ a unique and very useful differentiation quality/potency marker of chondrocytes as well as MSCs in tissue engineering of cartilage.

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Integrin α11β1: A Major Collagen Receptor on Fibroblastic Cells

5

Cédric Zeltz, Ning Lu, and Donald Gullberg

Abstract

Integrin α11 is the last addition to the vertebrate integrin family. In this chapter we will summarize some basic facts about this integrin and update with information that has been gained in the last decade. Integrin α11β1 is a major collagen receptor on a subset of fibroblasts. Extensive characterization of the expression pattern in developing mouse embryos has demonstrated expression restricted to subsets of fibroblasts and a transient expression in odontoblasts, but comprehensive characterization of corresponding expression in adult tissues is still lacking. Mice lacking integrin α11 are dwarfed, primarily due to defective incisor eruption defect, which can be traced back to need for α11 on periodontal ligament fibroblasts during incisor eruption. Separate studies have suggested reduced levels of IGF-1 in mice lacking α11. Analysis of lung cancer has identified α11β1 as a functional important collagen receptor on carcinoma associated fibroblasts (CAFs) and a number of disease models are awaiting analysis to see the importance of this collagen receptor in pathological models.

Keywords

Integrin α11 · Collagen · Fibroblasts · Carcinoma-associated fibroblasts · Myofibroblast

5.1 Introduction

Integrins are evolutionary old molecules that have been around for millions of years [11, 14], but we first identified integrin α11 in 1995 [12]. Integrin α11 is the last member of the integrin family to be discovered. This integrin subunit was initially named α_{mt}, since it was first identified on cultured human fetal myotubes. In parenthesis, this discovery was indeed serendipitous since the

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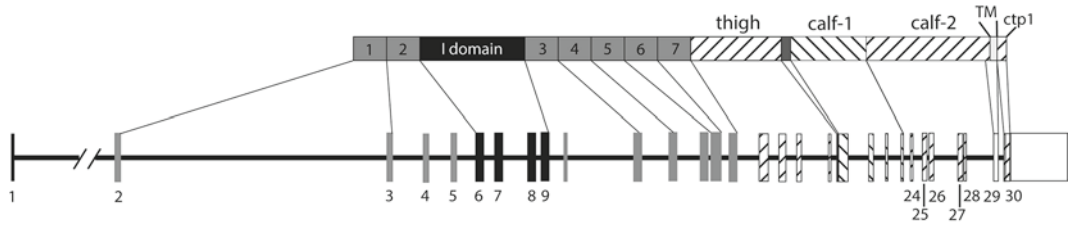

Fig. 5.1 Schematic representation of *ITGA11* gene. Top part shows a schematic representation of human α11 protein, and lower part shows a schematic overview of the organization of the *ITGA11* gene with its 30 exons. For the protein, the I domain, the 7 FG-GAP repeats (1–7), transmembrane part (TM) and cytoplasmic tail (ctpl) are marked. In the gene, exonic sequences representing untranslated regions are marked with open boxes

expression of α11 on myogenic cells is only seen upon tissue culture (in muscle tissue in vivo α11 is present on muscle fibroblasts). In 1999, we characterized this integrin and identified it as the β1-associated α11 subunit with properties of a collagen-binding integrin chain [26]. Two years later, we functionally described the α11β1 integrin as a collagen receptor involved in cell migration and collagen reorganization [24] and in 2004 we described the mouse variant of integrin α11 [21]. The generation of α11 integrin-deficient mice was a major advance in our efforts to elucidate α11 integrin function in health and disease [20].

5.2 The *ITGA11* Gene

The human α11 integrin gene (*ITGA11*) is localized on chromosome 15q23 and spans 130 kb, whereas the mouse α11 integrin gene (*Itga11*; length of 106 kb), has been mapped to chromosome 9. Both the human and mouse genes contain 30 exons and 29 introns (Fig. 5.1).

5.2.1 Promoter and Transcription Start

The *ITGA11* promoter lacks both TATA and CCAAG boxes. Promoters of other integrin α-chains contain features of a conserved initiator element often associated with an upstream Sp1 site found close to the transcription start [10, 32].

We used oligo-capping to identify a transcription start site (TSS) 30 nucleotides upstream of ATG in ITGA11 [30]. While the consensus sequence for initiator sequences is pypyANT/Apypy, the experimentally found sequence ACACC in *ITGA11* functions as an abbreviated initiator sequence. Furthermore an Sp1 binding site is located upstream of the putative initiator sequence, supporting the view that it is functional [18].

Using a panel of 15 serially-deleted promoter constructs, the α11 integrin proximal promoter spanning nt −176 and +25 nt has been characterized in the 3 kb *ITGA11* promoter region and found to convey high level of transcription activity. The presence of two Sp1 sites and an Ets-1 site in the proximal promoter is essential for its promoter activity [18] (Fig. 5.2).

Cytokines are able to regulate the α11 integrin expression by inducing signaling molecules, which regulate transcription factor binding to promoters. Thus, TGF-β1 was shown to up-regulate α11 expression in HT1080 fibrosarcoma cell line, as well as in human dermal fibroblasts and MRC-5 fibroblasts [13, 17]. The responsiveness to TGF-β1 is dependent on Smad2/3 and Sp1-regulated transcription. The Smad-binding element SBE2 and the Sp1-binding site SBS1 are closely located in the proximal promoter (nt –182/–176 and –140/–134, respectively). This proximity could promote a possible interaction between the Smad and Sp1 transcription factors. Activin A, which belongs to the TGF-β family, is involved in the up-regulation of α11 in mouse embryonic fibroblasts (MEFs), in a mechanosensitive manner [8]. This induction of α11 expression requires the Smad3 transcription factor. In transgenic reporter mice the human 3 kb *ITGA11* promoter drives a

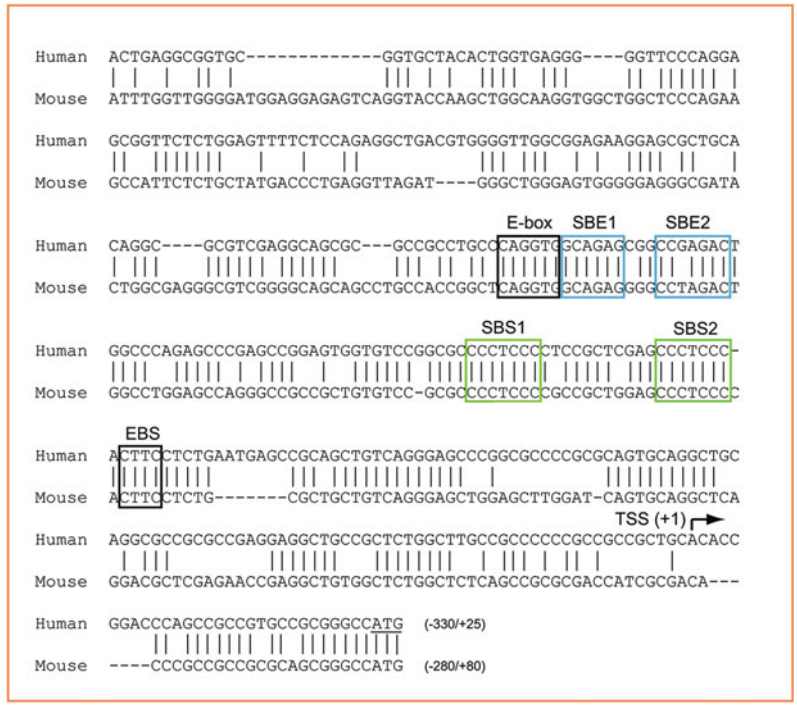

Fig. 5.2 Conserved promoter elements in human and mouse integrin α11 promoters. Alignment of the human integrin α11 promoter region −330 to +25 with the corresponding mouse promoter sequence. An E-box, two putative Smad binding elements (SBE1 and SBS2), tandem Sp1 binding sites (SBS1 and SBS2) and an Ets binding site (EBS) are boxed in the sequences. The human α11 transcription start site (TSS) is indicated by an *arrow* and the translation start codon ATG is *underlined*. From [17]

fibroblast-specific expression in developing mouse embryos (Fig. 5.3).

Type I interferons, including IFN-α and IFN-β, have also been described to regulate α11 expression. IFNs are able to stimulate α11 mRNA and protein expression in the glioblastoma derived cell line T98G [16].

Down-regulation of α11 has been reported to occur in mesenchymal stem cells and mouse embryonic fibroblasts treated with FGF-2 [8, 25]. However, the responsive elements involved in this down-regulation have not yet been determined in the α11 promoter.

5.2.2 Exon Structure

As in other integrin genes, the signal peptide is split by two exons, exon 1 and 2. Unlike some other integrin genes, exon 1 in *ITGA11* does not solely contain an UTR sequence, but also encodes the major part of the signal peptide, similar to the organization observed in the α10 integrin gene [4].

Previous analysis of the exon structure of leukocyte integrins has been very informative in identifying I domain borders. In the case of α11, the I domain is encoded by 4 exons (exons 6-9) and flanking introns are in phase 1.

We have previously identified an inserted region of 22 amino acids that distinguishes α11 from other integrin α chains [26]. It is likely that the inserted region has arisen by reshuffling of the existing sequence, which has then been inserted at the beginning of exon 20 during evolution. Comparison with *ITGA2* exon 20 (GenBank:AC016619.5) shows that the homologous sequence starts in a position immediately after the insert observed in exon 20 of *ITGA11*. Comparison of the α11 sequence with other integrin α-

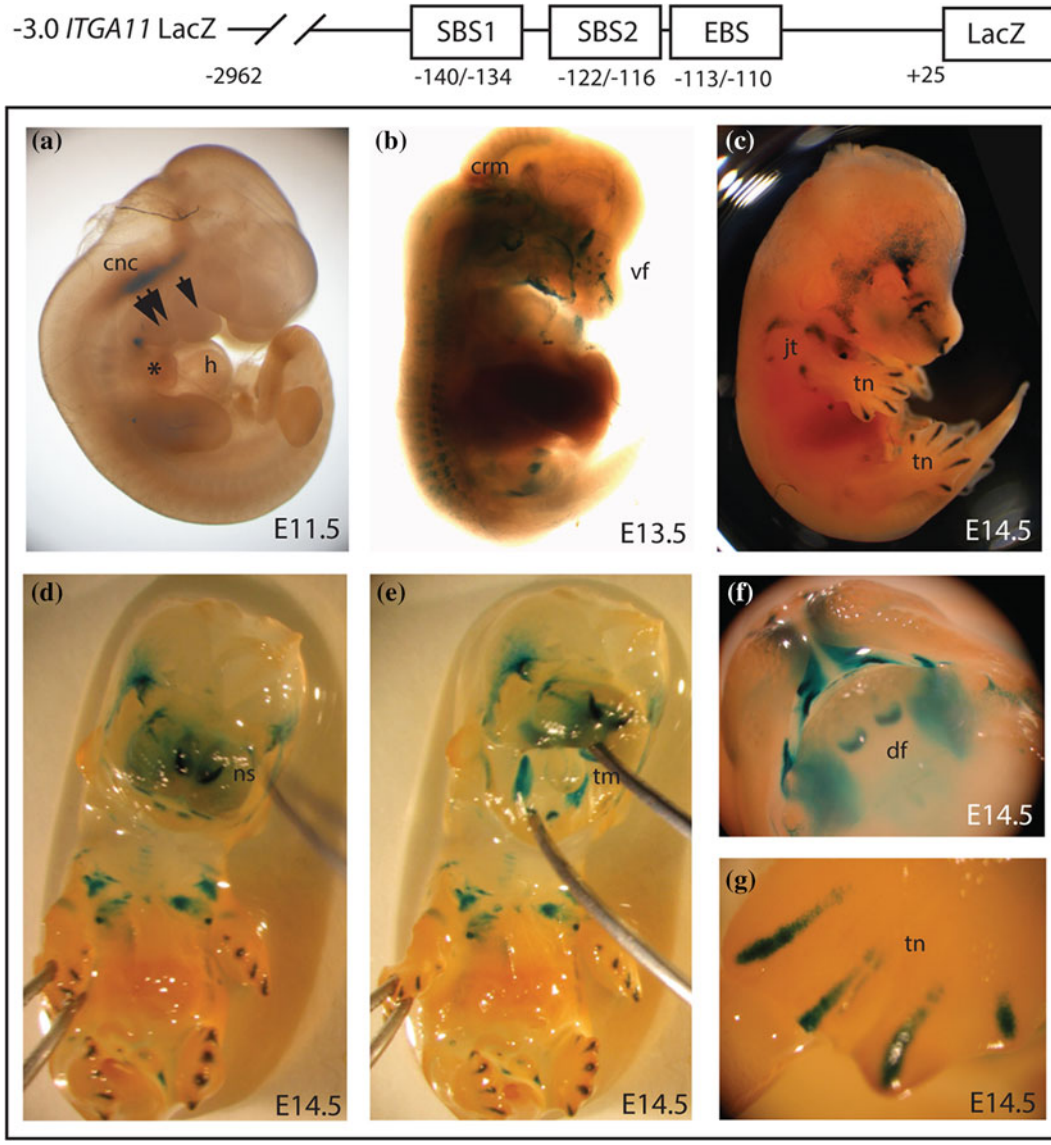

Fig. 5.3 A 3 kb ITGA11 promoter drives LacZ reporter expression in fibroblast precursors. The *upper* panel shows a schematic map of the −3.0 ITGA11promoter-LacZ reporter transgene used for generating transgenic mice. The previously identified Sp1 binding sites (SBS1 and SBS2) and an Ets family binding site (EBS) are indicated. The lower panel shows the whole-mount X-Gal staining of transgenic embryos at different embryonic days. LacZ expression is indicated by the blue staining. At E11.5 **a** LacZ expression was observed in the subpopulations of cranial neural crest cells cnc-cranial neural crest cells; in the first branchial arch which give rise to maxillary (*single arrow*) and mandibular (*double arrow*) prominences, and in the second branchial arch (*asterisk*) which will give rise to hyoid; h—heart; at E13.5 **b** LacZ expression was detected in *crm* cranial mesenchyme around calvarial bone, future location of the intervertebral disc and in *vf* vibrissae follicle; at E14.5 **c** LacZ expression was shown in *jt* joints, *tn* tendons of limbs, **d** in *ns* nasal septum, **e** in *tm* tongue mesenchyme, **f** in *df* dental follicles and **g** close up for forelimb showing positive staining in *tn* tendon (**g**). From [17]

chains places the insertion in a region of the stalk region called the calf-1 domain. Future studies will reveal the possible importance of this region. Interestingly, comparison with other integrins α-chains has identified this region as being involved in α/β chain interactions with the ability to influence integrin activation [28].

Careful examination of intron sequences using the program Genscan resulted in the identification of three potential in-frame exon sequences (with the tentative names 10B, 21L and 22B). We, however, failed to detect RNA messages for these variants, and currently no experimental data is thus available to support their existence. In *ITGA10* alternative splicing in a region corresponding to exon 25 has been described [4]. PCR amplifications of RNAs from different tissues covering this region in α11 transcripts suggest that corresponding splicing does not occur in α11.

The cytoplasmic tail in integrin α chains has a conserved sequence GFFXX, which in human α11 corresponds to the sequence GFFRS. It is interesting to note that for those integrin α chains that undergo alternative splicing in the cytoplasmic tail, the alternative exons also encode GFFKR, supporting the view that GFFKR together with the cytoplasmic tail denotes a functional unit. Biochemical analysis of the exact border of the transmembrane domain has recently suggested that GFFK residues are part of the transmembrane domain [1]. Comparison with the gene structure for α11 shows that if the homologous sequence GFFR is considered to be part of the transmembrane domain, the majority of this domain is encoded by exon 29, with the final four residues being encoded by the terminating exon 30, which also encodes the cytoplasmic tail.

5.2.3 Comparison with Other αI-Encoding Integrin Genes

Comparison of *ITGA10* and *ITGA11* genes lends further support to the view that α10 and α11 have arisen by a gene duplication event. Unlike *ITGA1* and *ITGA2*, which are both located on chromosome 5, *ITGA10* and *ITGA11* have been mapped to human chromosome 1 and 15, respectively. Unlike the *ITGA11* gene, which spans at more than 130 kb, *ITGA10* is much more compact and spans less than 19 kb. Comparison of *ITGA11* with the αX exon structure reveals a striking conservation of exon borders, underlining the close evolutionary relationship of integrin αI domain encoding genes.

The closely related *ITGA2* has been shown to display polymorphisms in the promoter region, also identified as risk factors for thrombotic disease [6, 7]. Based on the high expression of integrin α11 in the periodontal ligament (PDL) we hypothesized that single nucleotide polymorphisms (SNPs) in *ITGA11* might predispose to periodontitis. However, analyses of patients with juvenile periodontitis failed to identify polymorphism in the *ITGA11* basal promoter [3]. Further studies of the promoter will be instructive in determining: the regions in the upstream region that direct the fibroblast and carcinoma- associated fibroblast (CAF) -specific expression of α11 observed in vivo, the underlying mechanism for its mechanosensitivity and finally the regions mediating responsiveness to fibrogenic growth factors.

5.3 The Integrin α11 Subunit

ITGA11 encodes a mature protein of 1166 amino acids with a predicted integrin alpha chain structure. In SDS-PAGE, it runs as a 155kD band in a position above integrin α2 and α10 chains, indicating a higher degree of glycosylation than these two related integrins (Fig. 5.4). The extracellular domain contains seven FG-GAP repeats and a 195-amino acid-long I domain inserted between the repeats 2 and 3. The I domain presents a metal ion-dependent adhesion site (MIDAS) motif and three potential divalent cation binding motifs. As already mentioned the short cytoplasmic tail of 24 amino acids contains the motif GFFRS instead of the conserved GFFKR sequence most commonly found in integrin α subunits. A 23-amino acid-

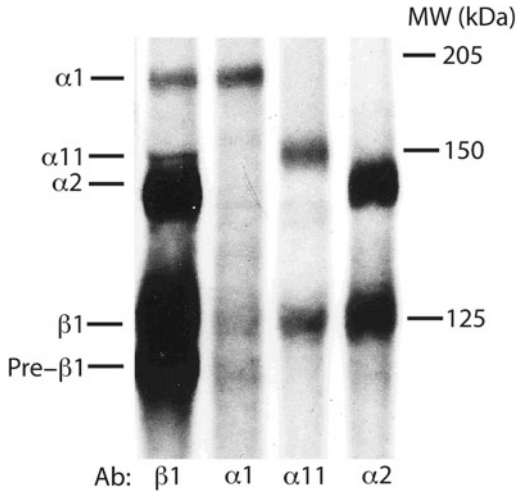

Fig. 5.4 Expression of α11 in cultured human fibroblasts. Cultures of subconfluent 1518 human foreskin fibroblasts were metabolically labeled, proteins were immunoprecipitated with antibodies, separated on a 6 % SDS–PAGE gel under nonreducing conditions, and visualized by fluorography. The antibodies used were directed to integrin subunits β1, α1, α11 and α2. Positions of different integrin chains are marked. From [24]

long transmembrane domain links the extracellular and cytoplasmic domains [26]. The mouse α11 integrin chain shows an 89 % identity with human α11 at the protein level and 97 % identity in the I domain [21].

5.3.1 Expression of the α11 Integrin Chain In Vivo

The expression of integrin α11 was first described in cultured human fetal muscle cells in vitro [12]. In human adult tissue, α11 mRNA was expressed in high levels in uterus and heart and in intermediate levels in skeletal muscle. However, in human and mouse embryos, no expression of α11 was detected in muscle cells [21, 24]. Later it was shown that in muscle tissue α11 is expressed in fibroblasts, hence explaining the detection of α11 RNA in muscle tissues in Northern blotting. α11 is present in fibroblasts around ribs, vertebrae, in intervertebral discs and in keratocytes of the cornea of 8-week human embryos [24]. In the mouse embryo, α11 has been localized to the ectomesenchyme in the head including the PDL, in tendons and intestinal villi fibroblasts [21]. The α11 chain expression appears to be specific to mesenchymal non-muscle cells in vivo (Fig. 5.5), but a complete characterization in adult tissues has not yet been performed. α11 expression has also been reported in tumor tissue from melanoma and lung carcinoma [27, 31]. The high levels of α11 integrin expression in lung carcinoma in situ are derived from the CAFs and is thus in the lung not contributed by the cancer cells. Recent data indicates that α11 RNA is regulated during epithelial mesenchymal transition [15].

5.4 Integrin α11β1 Functions

5.4.1 In Vivo Functions

The in vivo function of the α11 integrin has been partially elucidated using the knockout mouse model. The α11-deficient mice are smaller and display an increased mortality compared to heterozygous and wild-type mice [20]. Dwarfism observed in these α11-deficient mice appears not to be due to structural defects in forming cartilage or bone. Instead the smaller size and malnutrition of weaned α11-deficient mice appear to correlate with delayed incisor eruption and altered tooth shape (Fig. 5.6). The incisor PDL, which plays a central role during rodent incisor eruption, showed increased thickness due to increased amount of collagen. In this mutant tissue, a decrease of MT1-MMP and MMP-13 mRNA levels were also noted. A reproducible result was obtained in vitro, where MEFs isolated from α11-deficient embryos showed reduced MT1-MMP and MMP-13 mRNA expression, whereas MMP-2 and MMP-9 activities were not affected. These observations suggest that α11 could be involved in the regulation of metalloproteinases as MMP-13 and -14, thus controlling the collagen turnover in PDL. Later studies of PDL fibroblasts isolated from mouse incisors confirmed a role for integrin α11 β1 in regulating MMP-13 expression, but failed to show regulation of MMP-14 at the

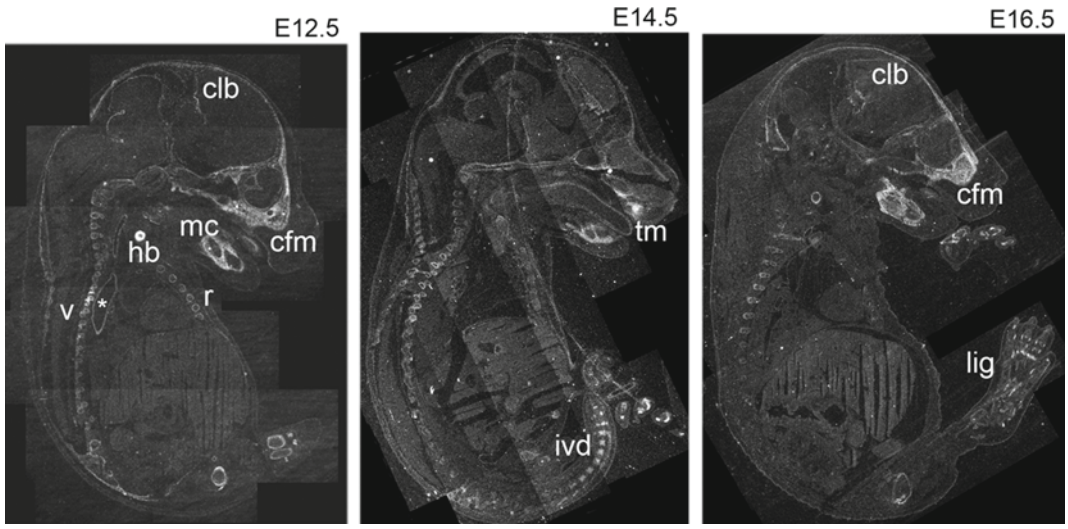

Fig. 5.5 Localization of α11 mRNA during mouse embryogenesis. Sagittal sections of mouse embryos from *E* embryonic days E12.5–E16.5 were subjected to in situ hybridization using an antisense RNA probe specific for mouse α11. Darkfield images are shown. At E12.5 α11 mRNA can be detected around *clb* calvarian bone, in *cfm* craniofacial mesenchyme, around *mc* Meckel's cartilage, around *hb* hyoid bone, around *v* vertebrae, and around the *r* ribs. * denotes signal in descending aorta, not confirmed by immunohistochemistry. At E14.5 α11 can also be detected in *ivd* intervertebral discs in the tail region and in *tm* tongue mesenchyme. At E16.5 tendons and *lig* ligaments in the hind limb express high levels of α11 RNA. From [21]

protein level under the conditions used [2]. Intriguingly, later data has shown that α11-deficient mice display reduced serum levels of IGF-1 [5]. The mechanism for how a fibroblast specific protein would affect the pituitary axis responsible for IGF-1 secretion is unclear at this stage. The study is however important since it stresses that α11-deficient mice are smaller already at birth, before the incisor eruption effect on body weight has come into play and further stresses that more detailed studies are needed to sort out the underlying molecular mechanism for reduced body weight observed in α11-/- mice.

As described above, α11β1 has been reported to be up-regulated in the CAFs in non-small cell lung cancer [31] (Fig. 5.7) and was also in a xenograft model shown to enhance tumorigenicity by regulation of the IGF-2 expression. However, the exact role of α11 in the tumor stroma during TGF-β1-dependent myofibroblast differentiation, tumor growth and tumor metastasis remains to be determined and it will be important to determine if α11 upregulation occurs on CAFs also in other types of tumors.

5.4.2 In Vitro Functions

The α11 integrin chain is exclusively associated with the β1 subunit at the cell surface, to form the α11β1 integrin. The first study that demonstrated that α11β1 promoted cell attachment to collagen I was performed in 2001, when we started using the system of transfecting cDNAs encoding collagen-binding integrins into the mouse satellite cell line C2C12 [24]. Using these cells we could show that integrin α11β1 displays certain collagen specificity, since it binds preferentially type I collagen, whereas it interacts with collagen IV with a low affinity. The α11 I domain recognizes the triple-helical GFOGER sequence present in collagen I as well as the GLOGER motif [22, 29].

Another study has identified the GLPGER motif of the recombinant Scl1 protein, a prokaryotic collagen, as an α11β1 binding sequence [9]. The interaction between the cell surface streptococcal Scl1 and the human α11β1 integrin might increase host colonization by pathogenic bacteria, but this process remains to be determined.

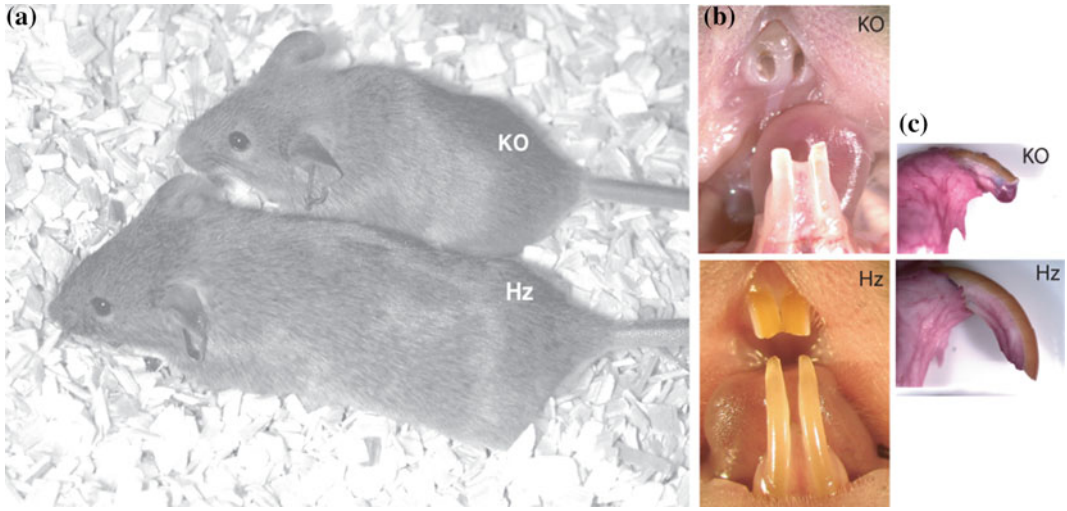

Fig. 5.6 Phenotype of integrin α11-deficient mice. **a** 10-week-old male mutant mice showed a reduction in size. Hz, heterozygous ($\alpha11^{+/-}$); KO, homozygous ($\alpha11^{-/-}$). **b** A lack of the outer portion of the upper incisors was observed in the α11 null mice at 1 year of age. **c** The incisors of 4-month-old mutant control (*lower panel*) and mutant (*upper panel*) mice were excised from their sockets, and the soft tissue was digested away. Note the altered size and shape of the KO incisors. From [20]

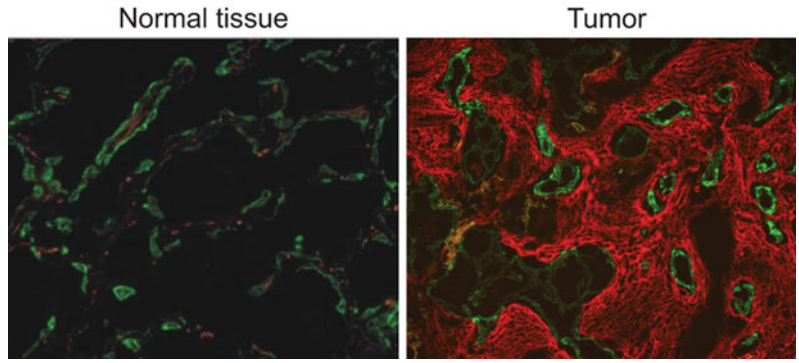

Fig. 5.7 Expression of integrin α11 chain in tumor stroma. Immunofluorescence images of human Normal tissue (lung) and Tumor tissue (non small cell lung adenocarcinoma) that were double stained with antibodies to α11 (*red*) and epithelial cell marker cytokeratin (*green*). The α11 staining was negligible in non-neoplastic lung tissue and was mainly confined to the stroma in the tumor sample. From [31]

The role of $\alpha11\beta1$ in PDGF-stimulated cell migration on collagen I coating seems to be cell type dependent. The C2C12 mouse cells, stably transfected with human α11 integrin cDNA, showed a stronger chemotactic response to PDGF-BB, compared to C2C12 wild-type cells, lacking endogenous collagen receptors [24]. In contrast, MEFs depleted in $\alpha11\beta1$ migrated more on collagen I in comparison to wild-type embryonic fibroblasts [21]. However, in this last case, a compensatory mechanism, involving other collagen receptors, cannot be excluded.

In several studies we have shown that $\alpha11\beta1$ mediates the contraction of collagen lattices, an important function, which contributes to the regulation of the reorganization of collagen matrices [2, 3, 20, 24]. Interestingly, when we isolated PDL fibroblasts from α11 deficient mice, these fibroblasts displayed reduced levels of MMP-13 and cathepsin K, which in $\alpha11\beta1$

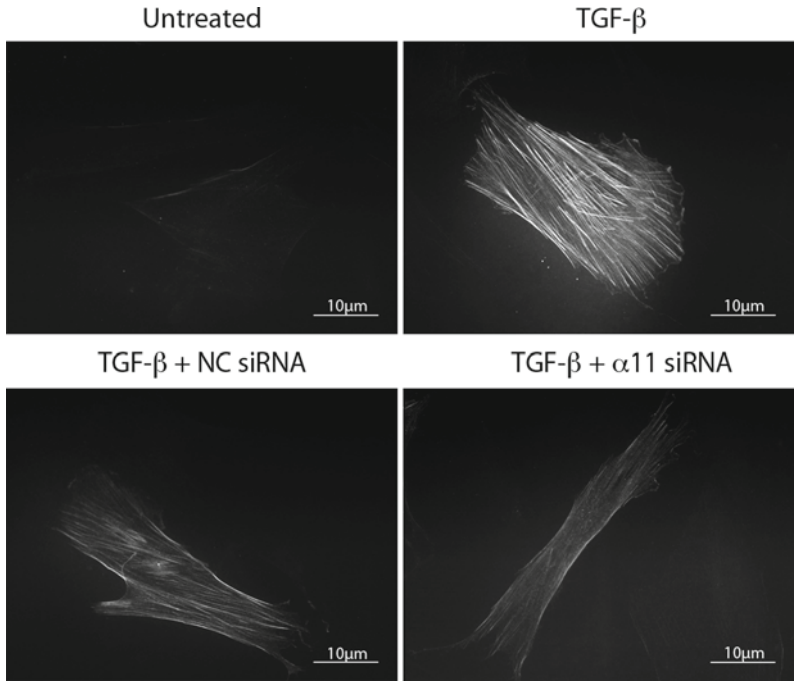

Fig. 5.8 α11 influences myofibroblast differentiation in human corneal fibroblasts. Immunolocalization of α-SMA in corneal fibroblasts that remained untreated (*upper left*), were stimulated with 5 ng/ml TGF-β1 only (*upper right*), or were treated with 5 ng/ml TGF-β1 and α11-specific siRNA (100 nM) (*lower right*), or an off-target siRNA (*NC* negative control; *lower left*). The exposure time when acquiring pictures was identical in all conditions. From [8]

expressing fibroblasts seemed to facilitate the collagen remodeling process during collagen contraction [2].

Under certain conditions fibroblasts become activated and differentiate into so-called myofibroblasts. Myofibroblasts are characterized by α-smooth muscle actin (α-SMA) incorporated into stress fibers. Corneal fibroblasts, under action of TGF-β1, overexpress α-SMA. Since siRNA directed against the α11 integrin completely abrogated α-SMA up-regulation, these data demonstrate that α11β1 also plays a role in myofibroblast differentiation [8] (Fig. 5.8). The regulation of myofibroblast differentiation by α11β1 could be relevant in pathological processes such as tumor-stroma interactions and fibrosis, where myofibroblasts are involved (Fig. 5.9).

More recently α11- and TGF-β2-dependent myofibroblast differentiation in cardiac fibroblasts has been observed, suggesting a potential role for α11 in cardiac fibrosis [23].

Integrin turnover is an essential process involved in cell adhesion and migration. Generally, integrins present on the cell surface are either released and used in new adhesion sites or internalized by endocytosis. Rab proteins, including Rab21, regulate the traffic of endocytotic vesicles via interaction with the cytoplasmic tail of α integrin subunit, as shown for integrin α2β1 [19]. The C-terminal part of Rab21 was also able to bind to the cytoplasmic domain of α11 integrin, thus suggesting that α11β1 could be regulated by endocytosis. Since Rab21 activity has been shown to regulate the motility of breast and prostate cancer cells, it could be interesting to examine if an association between this small GTPase and α11β1 occurs in cancer-associated fibroblasts and if it might have an impact on the tumor progression.

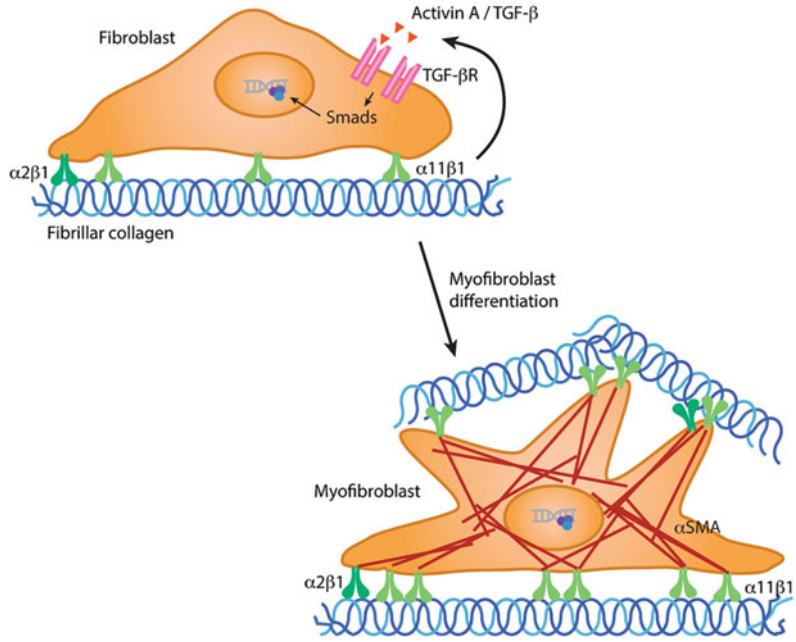

Fig. 5.9 Schematic illustration of role of integrin α11 in myofibroblast differentiation. Under mechanical stress, on a stiff substrate, fibroblasts secrete members of TGF-β , influenced by $\alpha 11\beta 1$ integrin. TGF-β signaling, via Smad proteins, induces fibroblast differentiation into myofibroblast by up-regulation of α-SMA and $\alpha 11\beta 1$ expression. Myofibroblasts synthesize collagen and are able to reorganize collagen matrices in an $\alpha 11\beta 1$-dependent manner. We hypothesize that the regulation of myofibroblast differentiation by $\alpha 11\beta 1$ is relevant in fibrosis and tumor-stroma interactions

5.5 Perspectives

Integrin $\alpha 11\beta 1$ is expressed in mesenchymal non-muscle cells in vivo at sites where collagens are organized in a highly ordered manner. It appears as a multifunctional integrin in different contexts. However, little is known about the detailed molecular mechanisms involved in $\alpha 11\beta 1$ functions including the major signaling pathways utilized by $\alpha 11\beta 1$ and its involvement in various pathological conditions, and thus much still remain to be learned about this collagen receptor.

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Regulation of Integrin Activity by Phosphorylation

6

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Abstract

Integrins are heterodimeric complex type I membrane proteins involved in cellular adhesion and signaling. They exist as inactive molecules in resting cells, and need activation to become adhesive. Although much is known about their structure, and a large number of interacting molecules have been described, we still only partially understand how their activities are regulated. In this review we focus on the leukocyte-specific $\beta 2$—integrins and, specifically, on the role of integrin phosphorylation in the regulation of activity. Phosphorylation reactions can be fast and reversible, thus enabling strictly directed regulatory activities both time-wise and locally in specific regions of the plasma membrane in different leukocytes.

Keywords

Integrin · Phosphorylation · Leukocyte · Adhesion · Signaling

6.1 Introduction

About 30 years ago leukocyte cell surface proteins were described to be involved in various functions such as antibody production, cytotoxicity, phagocytosis and chemotaxis [1, 2]. They were given names such as leukocyte function associated antigen (LFA-1) and macrophage antigen-1 (Mac-1). Later work used phorbol esters to induce cell adhesion. In the presence of an array of monoclonal antibodies reacting with the leukocyte surface we looked for antibodies, which could inhibit the induced adhesion. One antibody, called 60.3, was efficient, and immune precipitation resulted in the identification of protein dimers [3, 4]. Subsequently, these proteins were shown make up a subfamily of adhesion proteins named integrins [5, 6]. Because of their functional importance, they drew large interest and the literature on integrins is currently impressively large [7–11].

Integrins are present in the animal kingdom from nematodes and fruit flies to humans, and their amino acid sequences are remarkably well conserved [10, 12]. The ligand binding domains

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on the outside of cells are large, and some integrins, including the β2-integrins, contain an I/A-domain. When present, the I-domain first characterized in von Willebrand factor, forms the ligand binding site and is located in the α-chains of the integrins. In humans four β2-integrins exist. The lymphocyte enriched LFA-1 (αLβ2, CD11a/CD18) binds to the intercellular adhesion molecules (ICAM) −1 to −5 [13–15], but the other family members Mac-1 (αMβ2, CD11b/CD18), αXβ2 (CD11c/CD18) and αDβ2 (CD11d/CD18) bind to several additional types of ligands including plasma proteins, extracellular matrix components and even carbohydrates. In integrins lacking the I-domain, the ligand binding site is formed by the β-propeller domain on the α-chain and the βI-domain of the β-subunit.

The β2 integrins are expressed on white blood cells, but the expression profile on different leukocytes is unique for each member of the family. LFA-1 is expressed on all leukocytes, whereas αMβ2 is found on monocytes, macrophages, NK cells, neutrophils and on $\gamma\delta$ subsets of T cells. αXβ2 is expressed on monocytes, macrophages, dendritic cells, NK cells and some subsets of T and B cells, and αDβ2 on macrophages and eosinophils [8, 10] The functions of leukocyte integrins are vital for a functional immune system, but, reflecting the variance in the expression pattern, also the functions of the family members are somewhat different. Leukocyte integrins and especially αLβ2 are essential for the extravasation of the immune cells from the circulation to inflamed tissues. αLβ2 is also necessary for the proper formation of the immunological synapse that forms between an antigen presenting cell and a T cell [16]. The complement receptors αXβ2 and especially αMβ2 have been shown to be important in phagocytosis. Other functions of αMβ2 include remodeling of ECM, survival of neutrophils and development of immune tolerance [17, 18]. αXβ2 on the dendritic cells has been found to be an efficient target for antigen uptake in eliciting T cell immune responses [19]. Other studies have demonstrated the role of αXβ2 on hypercholesterolemic mouse monocytes in the development of atherosclerosis [20]. αDβ2 on macrophages possibly takes part in phagocytosis and migration [18].

The integrins are remarkable in the sense that they can signal across the plasma membrane in two directions: inside–out and outside–in. In the former case, a ligand or activating agent is bound to a non-integrin receptor and eventually the signal is transmitted to the integrins. In outside–in signaling the ligand binds directly to the integrin on the outside of the cell and induces signaling. Increasing evidence shows that inside–out signaling results in partial activation of the integrins where the head piece remains closed and the integrin weakly binds to the ligand. When the ligand binds, the binding region in the integrin opens up followed by outside–in signaling. Thus inside–out and outside–in signaling may be coupled and occur subsequently in an individual integrin.

6.2 Integrin Structural Features

Figure 6.1 shows schematic figures of leukocyte β2-integrins [9]. The different domains are schematically drawn in Fig. 6.1a. The external domains are complex and interact non-covalently on the outside of the cell. A major advance in understanding integrins came when the I-domain from αM was crystallized and the structure determined [21]. Later the structures of the external parts of the αVβ3 and the αXβ2 integrins were determined [22, 23]. Surprisingly, the binding site formed by the α-chain propeller and the β-chain I-like domain was turned towards the lipid membrane (Fig. 6.1b). Later work showed that upon activation the molecules straighten out and now show intermediate binding affinity (Fig. 6.1c). In the fully active protein, the binding site has opened up, the hybrid domain has moved out and presumably the "legs" including the cytoplasmic tails have moved apart (Fig. 6.1d). All results indicate that the integrin α- and β-subunits play very different

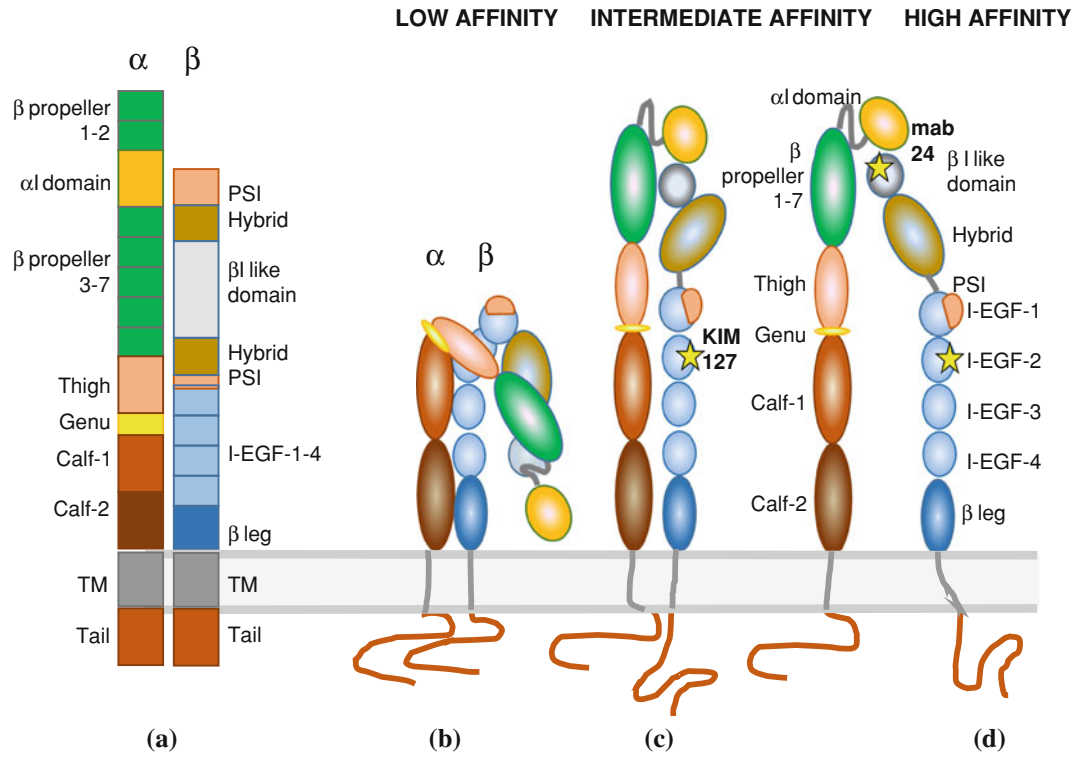

Fig. 6.1 Schematic figure of the αLβ2 integrin. **a** The domains are depicted. **b** In the resting state the integrin head piece containing the binding site is turned towards the membrane. **c** The integrin of intermediate affinity is straightened out, but the head piece remains closed, and the cytoplasmic domains are more closely packed. The binding site of the KIM127 antibody, which recognizes an integrin of intermediate affinity is shown. **d** The fully activated integrin. Note that the legs, the transmembrane domain and the cytoplasmic parts have moved apart, and the binding site in the I-domain is open. The location of the binding site of antibody mab24, which only binds to fully activated integrins is shown

roles in adhesion. The α-chains have an important structural role, including ligand specificity, but not necessarily a regulatory role. The β subunit cytoplasmic domains, on the other hand, are largely conserved and are able to regulate integrin activity. It has been proposed that in the resting state, a salt bridge exists between an aspartate in the β-subunit (SDLR in β2) close to the membrane, and the arginine in the conserved α-chain GFFKR sequence [24, 25]. Upon activation, this salt bridge may be broken enabling a switch from a relatively parallel heterodimer to a more X-like structure, which reaches out and modifies the structure also on the outside of the membrane. How this could take place is discussed below.

6.3 The Integrin Cytoplasmic Domains Bind Several Intracellular Regulatory Proteins

Much work has focused on the cytoplasmic domains of the integrins (Fig. 6.2). We now know that the cytoplasmic regions of the integrins are pivotal in the regulation of activity. The structure of the cytoplasmic tails of αLβ2, αMβ2 and αXβ2 [26–28] as well as those of αIIb [24] and α4 [29] have been solved by NMR. The α-chains of β2-integrins show quite striking differences, even though the membrane proximal structures, forming a conserved helical structure,

Fig. 6.2 The cytoplasmic sequences of the β2-integrins and those of some other integrin subunits dealt with in the text are shown. The functionally important α-chain regions, the potential phosphorylation sites, the NPXY sequences and other possibly important amino acids are *shaded*. Confirmed phosphorylation sites are numbered

remain similar. All leukocyte integrin α and β cytoplasmic tails studied appear to interact with each other through multiple ionic and hydrogen bonds in the membrane proximal helical area, but the rest of the cytoplasmic α-chains form different structures. αL, which has the longest cytoplasmic part, forms three alpha helices that are sustained by salt bridges and/or hydrogen bonds. This structure forms a large negatively charged surface that is able to bind metal ions [26]. The membrane distal residues of two other leukocyte integrins, αM and αX, form loops that can adopt more conformational variations. These findings suggest that the different members of the leukocyte integrin family may have different cytoplasmic binding partners, which can lead to

different signaling events and outcomes. The reported phosphorylation sites of αL, αM and αX cytoplasmic tails as well as the β2 tail are situated outside the membrane-proximal helices and are thus more easily available for kinases and phosphatases involved in phosphorylation as well as for other cytoplasmic molecules for interactions. There are several recent articles and reviews dealing with the binding of cytoplasmic proteins to integrin intracellular domains [12, 30–37] and we will not try here to cover all aspects of this fascinating, but large subject. Instead, we focus on how integrin activities can be regulated rapidly and specifically. We want to put forward the proposition that integrin regulation primarily takes place through specific phosphorylation reactions, which in turn affect the binding of specific adaptor proteins. Further downstream binding components then relay various integrin related cellular functions.

Few cytoplasmic proteins have been found to specifically bind to the integrin α-chain cytoplasmic tails. These include paxillin, calreticulin, CD45, RapL and the adhesion inhibitory protein SHARPIN [38]. In contrast, more than 40 proteins have been claimed to bind to the β-subunits [35]. The integrin β-chain cytoplasmic regions contain three important "hot spots" for binding proteins. These are the two NPL(I)Y(F) and N(XX)Y(F)(or NPXY for short) sequences and the Ser/Thr enriched sequence between them. Depending on the integrin, all three sequences are also potential phosphorylation sites. It should, however, be pointed out that several integrin cytoplasmic domains have been used for studies on interactions with cytoplasmic proteins, and certainly different integrins have different binding preferences. Therefore, results obtained with one cytoplasmic sequence must not necessarily be true for another one. Furthermore, the cytoplasmic parts of the integrins are relatively short with a limited binding capacity. Obviously, the interactions are competitive to a large extent and binding of a given cytoplasmic protein often excludes the binding of another one.

The best studied cytoplasmic proteins interacting with integrin β-chain COOH-terminal regions are talin, filamin, kindlins and 14-3-3 (Fig. 6.3). The talin molecule is large with a "head" and a long "tail" (rod). The head binds to integrins whereas the tail may interact with the actin cytoskeleton, but also with integrins [39]. The properties of talin and its interactions with integrins have been described in several recent reviews [12, 30, 32, 36, 39]. Importantly, the integrin β-subunits have two binding sites for talin, an acidic sequence close to the membrane [40], and the proximal NPXY(F) sequence. Filamin binds to a region extending from the first to the end of the second NPXY(F) sequence whereas kindlins bind to the region beginning from the Ser-Thr enriched region (Thr-Thr-Thr in β2) and covering the second NPXY(F) sequence. The 14-3-3 proteins bind to Thr-758 in the Thr-Thr-Thr sequence in β2, but only after phosphorylation [41, 42]. A weaker binding may also occur, which is not dependent on phosphorylation [12].

6.4 Integrin Phosphorylations are of Pivotal Importance for Activity

An accumulating amount of recent results indicate that specific integrin phosphorylations are important in the regulation of integrin associated activities [43]. The leukocyte integrins have turned out to be especially useful models for studies on phosphorylation mediated regulation. In contrast to other cells, blood cells such as lymphocytes, are normally completely resting, but can be activated by various agents including chemokines and lectins, but also through the T cell receptor. In addition, immunologists have obtained a vast knowledge of leukocyte functions. Certainly, there exists a large clinical interest in leukocytes and in several diseases in which leukocytes play an important role. These facts have further stimulated work on leukocyte integrins.

The early finding that integrins can be activated by phorbol esters, which were known to activate protein kinase C isoenzymes, led to studies on integrin phosphorylation. It soon turned out that the α-chains are constitutively

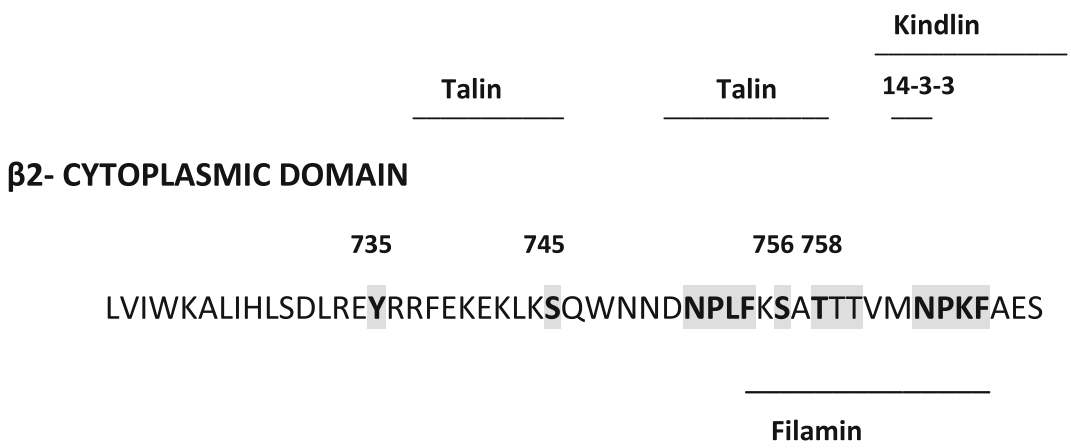

Fig. 6.3 The cytoplasmic domain of β2. The phosphorylation sites are numbered, and the functionally important amino acids shaded. The binding regions of some major cytoplasmic proteins binding to the β2-integrin cytoplasmic domain are indicated. Note that talin has two binding sites in β2

phosphorylated, whereas the β-chains only became phosphorylated upon activation [44, 45]. The leukocyte β2-integrins and the platelet αIIb/β3 integrin have been best studied in this respect. The possible and identified phosphorylation sites in the most studied integrin subunits are shown in Fig. 6.2. The αL, αM and αX subunits have single phosphorylation sites, all on serine residues. In contrast, the β-chains contain several phosphorylation sites. In β2, phosphate has been found on Ser-745, Ser-756, Thr-758, Thr-759, Thr-760 and possibly on Tyr-735 [41, 46]. αL is phosphorylated on Ser-1140, αM on Ser-1126 and αX on Ser-1158 [47–49]. In human β1 and β3 integrin subunits, both NPXY sequences can be phosphorylated, but not in β2 where the tyrosines are replaced by phenylalanines. β7 is similar to β2 in that the first threonine in the threonine triplet is phosphorylated [46].

In addition to the protein kinase C family enzymes known to be responsible for integrin β2-chain phosphorylations [41, 50] other Ser/Thr kinases may be important. Ser-756 is strongly phosphorylated upon phorbol ester treatment, and it seems possible that it is phosphorylated by calcium/calmodulin kinase II, because the antagonist W7 inhibits the phosphorylation [51]. In addition, the integrin linked kinase (ILK), and the mammalian sterile20-like1 kinase (Mst1) have been implicated in integrin regulation [52, 53]. Also protein kinase A may participate in integrin related intracellular communication [54]. The p21-activated kinase 4 (Pak 4) phosphorylates Ser-759 and Ser-762 in the integrin β5 subunit [55]. This is proximal to the Ser/Thr phosphorylation site present in several β-chains. Mutation of the serines in this SERS sequence reduced the migration of the αVβ5-containing cells .

When T lymphocytes are activated through the T cell receptor, the inside-out signaling results in phosphorylation of Thr-758 in the β2 subunit of αLβ2 [46]. The phosphorylated β2-chain now binds 14-3-3 proteins, which are dimers with two Ser/Thr-phosphate binding domains [56]. This is followed by binding of the adaptor protein Tiam1 [57], followed by activation of the small G protein Rac-1 by Tiam1 [47, 58], (Fig. 6.4). Importantly, the phosphorylation signaling from the integrin β2-chain can be mimicked using a membrane permeable peptide containing phosphate at Thr-758. This peptide, when introduced into lymphocytes, was able to activate the pathway through the β2-chain resulting in increased adhesion [47]. Filamin is bound to the β2-chain in resting cells, but phosphorylation of Thr-758 results in a switch from filamin binding to 14-3-3 binding.

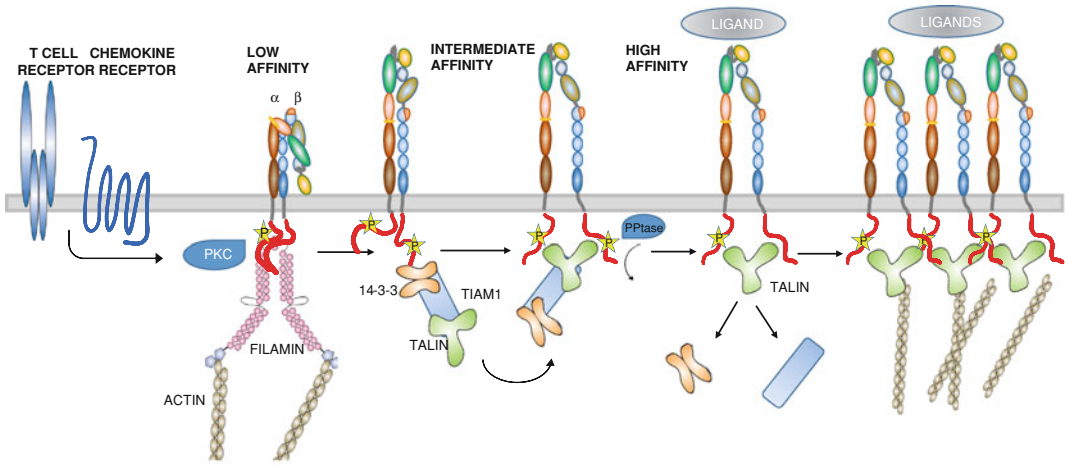

Fig. 6.4 Signaling from the T cell receptor/chemokine receptor resulting in active integrins. Ligand binding to the receptors results in activation of protein kinase C,s (PKC). This leads to phosphorylation of Thr-758 in the integrin $\beta 2$ subunit. The cytoplasmic tail of the integrin is released from filamin and 14-3-3 proteins bind to the $\beta 2$ chain, and the protein straightens out. A complex of the adaptor protein Tiam1 and talin is recruited to the integrin. The phosphate is subsequently released by phosphatase activity and talin can now directly bind to the integrin and activates it to high affinity. The interaction of talin with the actin cytoskeleton further increases the cellular binding to ligands by clustering of the integrin molecules resulting in increased avidity

Surface plasmon resonance experiments showed that the affinity of 14-3-3 proteins for phosphorylated $\beta 2$ binding is high whereas filamin showed no binding [42]. Talin can in fact bind both to the unphosphorylated and phosphorylated chains, but its binding to the phosphorylated molecule is competed out when 14-3-3 is present. The structural explanation for 14-3-3 and filamin binding to the $\beta 2$ cytoplasmic fragment has been determined. The phosphate on Thr-758 interacts electrostatically with Arg-56 and Arg-127 and by a hydrogen bond to Tyr-128 in 14-3-3. The filamin pocket in domain 21 can accomodate the unphosphorylated $\beta 2$ peptide, but after phosphorylation there is no room for the peptide with the hydrophilic phosphate [42].

Less is known about the effect of phosphorylation on Ser-756. Mutation of it to methionine resulted in inhibition of phagocytosis of C3bi-coated erythrocytes [17, 59]. The small G protein Rap1 was shown to bind to the phosphorylation mimicking Ser-756/Asp, but not to the non-phosphorylatable mutant [59].

Relatively little is known the connection between outside-in signaling and phosphorylation. $\alpha L\beta 2$ can be activated by integrin binding activating antibodies [60] soluble ligands such as ICAM-2 [61] and a peptide from the external part of ICAM-2 [62]. Ser-745 in the $\beta 2$-chain was found to be phosphorylated by soluble ICAM-2. This phosphorylation resulted in the release of the transcription co-activator JAB-1 from the $\beta 2$-chain enabling its downstream signaling to the nucleus [63].

The α-chain phosphorylations have been shown to be important. When αL, αM or αX, which are phosphorylated at positions Ser-1140, Ser-1126 and Ser-1158, respectively, are mutated with non-phosphorylatable alanines, adhesion was abrogated [47–49]. In $\alpha X\beta 2$ the α-chain phosphorylatable residue could be replaced by aspartic acid regaining adhesion [49]. Whether this is true for the other $\beta 2$-integrins is not known. A large proportion of the α-chains are constitutively phosphorylated, but there is still a continuous turnover of the phosphate [64]. Interestingly, outside-in activation by an integrin activating antibody resulted in activation of the Syk kinase both in wild type $\alpha X\beta 2$ and αXSer-1158/Ala transfected cells, but adhesion was blocked in the Ser-1158/Ala transfected cells [49]. The fact that adhesion was normal in

Ser-1158/Asp transfected cells shows that the negative charge on residue-1158 is important, but it must not be phosphate. Furthermore, when cells were transfected with the constitutively active small G protein Rap1, wild type cells adhered but cells with the Ser-1158/Ala mutation did not [49].

The αM cytoplasmic domain is the shortest of the β2-integrin α-chain cytoplasmic domains and it is phosphorylated on Ser-1126 (Fig. 6.2). Interestingly, the corresponding amino acid in the other α-chain domains is glutamic acid. This could mean that the negative charge at this position is important for activity, but it can be either phosphate or amino acid based.

Tyrosine phosphorylation is important in signaling events such as those taking place downstream from the T cell receptor and it may involve a number of kinases and substrates [65]. Src family tyrosine kinases (Src, Lck, Fyn etc.) could be responsible for integrin tyrosine phosphorylation, but further details are poorly known [43]. Integrin tyrosine phosphorylation has been shown to take place upon ligand binding to the αIIb/β3 integrin [66]. This integrin contains tyrosines in the two NP(I)XY sequences and they become phosphorylated when platelets bind to ligands such as fibronectin, fibrinogen and von Willebrand factor [66–69]. In β1-integrins similar tyrosine phosphorylations occur and these may contribute to the phenotype of transformed cells. When the tyrosines were mutated to phenylalanines, cell movement was inhibited [70, 71]. Importantly, tyrosine phosphorylation of β1 has been shown to inhibit talin binding to the proximal NPXY sequence leaving room for other proteins to bind, for example filamin and tensin [72]. The integrin β2-subunit does not contain tyrosine in the NPXY sequences but phenylalanine. Evidently, the β1- and β2-integrins are regulated differently, in both cases by phosphorylation, but it can be either on Ser/Thr residues or on tyrosine. Interestingly, the β2 subunit has tyrosine at position 735 and mutation of this residue impairs integrin recycling [73]. In contrast, the β1- and β3-subunits contain phenylalanine in the corresponding position. Little is known about tyrosine phosphatases in this connection. The CD45 tyrosine phosphatase has been implicated in the regulation of T cell signaling probably by activating Src family kinases by removing the COOH-terminal phosphate. Interestingly, CD45 has been found to bind to integrin α-chains and it could be part of a signaling complex and the immunological synapse [16].

6.5 How Does Integrin Phosphorylation Affect Talin Binding?

How does talin and kindlins fit into the story on phosphorylations? Talin has been claimed to be the final activator of integrins. Using nanodiscs with a single lipid-embedded integrin Ye et al. [36] showed that purified talin could activate the αIIbβ3 integrin. Although several details are still incompletely understood, we propose the following model. Upon activation through the T cell receptor or through chemokines, phosphorylation takes place on Thr-758 in the β-subunit. This results in release of filamin from the β-chain and replacement by 14-3-3 proteins. The 14-3-3 proteins are homodimers and the free binding site binds in turn to Tiam1 and activates the small G protein Rac-1 resulting in remodeling of the cytoskeleton [57]. On the other hand, it is possible that Tiam1 binds to talin [32] and after dephosphorylation talin could bind to the integrin β-subunit. Kindlin-3 has been shown to bind to threonines-758-760 and the distal NPKF sequence [31, 74]. Probably, phosphorylation of Thr-758 inhibits the binding, which can be restored after dephosphorylation.

The negative charge on the integrin α-subunit due to phosphorylation, combined with recruiting of the β-chain-14-3-3-Tiam1-talin complex to the actin cytoskeleton after activation, could induce breaking of the bonds between the α- and β-subunits resulting in separation of the integrin tails. The talin head domain could then

intercalate between the subunits. Serine/threonine phosphatase(s) like PP1 could remove the β2-chain phosphate enabling talin and kindlin binding to the β-chain. The talin and kindlin associations with the β-subunit would result in an allosteric change in the integrin resulting in activation of the molecule across the membrane.

6.6 Inhibition of Adhesion

Recently, natural inhibitors of integrins have been reported. The Del-1 protein is deposited on the surface of endothelial cells and binds to $\alpha L\beta 2$ and $\alpha M\beta 2$ integrins [75]. Evidently, it competes with the ICAM-molecules for integrin binding, and due to the fact that it is a soluble protein with relatively low affinity to integrins, it does not support leukocyte adhesion in vivo, but instead it inhibits binding by interfering with integrin/ICAM binding. Whereas Del-1 binds to the external surface of leukocytes, the intracellular protein SHARPIN binds to the cytoplasmic part of integrin α-chains and inhibits integrin activation [38, 76]. The SHARPIN/integrin interaction may compete out other α-chain cytoplasmic protein interactions, such as that of paxillin or inhibit the interaction of α-chains with the integrin β-chains.

6.7 Concluding Remarks

Protein phosphorylation is certainly extremely complex and because of its transient nature difficult to study. On the other hand it is fascinating to see how this relatively small protein modification can induce remarkable cellular changes enabling fast and precise adjustments in the highly variable environments of circulating and more stationary leukocytes. It is possible that the large number of protein kinase (>500) and phosphatase (>100) coding genes in higher organisms may forever preclude a detailed understanding of integrin function. But we optimistic and believe that a stepwise well defined approach can reveal the inner secrets of cellular adhesion complexity.

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Integrin αEβ7: Molecular Features and Functional Significance in the Immune System

7

Gregg A. Hadley and Jonathan M. G. Higgins

Abstract

Alpha E beta 7 (αEβ7) is an α-I domain-containing integrin that is highly expressed by a variety of leukocyte populations at mucosal sites including intraepithelial T cells, dendritic cells, mast cells, and T regulatory cells (Treg). Expression depends largely or solely on transforming growth factor beta (TGF-β) isoforms. The best characterized ligand for αEβ7 is E-cadherin on epithelial cells, though there is evidence of a second ligand in the human system. An exposed acidic residue on the distal aspect of E-cadherin domain 1 interacts with the MIDAS site in the αE α-I domain. By binding to E-cadherin, αEβ7 contributes to mucosal specific retention of leukocytes within epithelia. Studies on αE knockout mice have identified an additional important function for this integrin in allograft rejection and have also indicated that it may have a role in immunoregulation. Recent studies point to a multifaceted role for αEβ7 in regulating both innate and acquired immune responses to foreign antigen.

Keywords

Integrins · Intraepithelial T cells · Regulatory T cells · Dendritic cells · Mast cells · TGFβ

7.1 Introduction

Integrin αEβ7 is, in many respects, an unusual integrin. The αE subunit (CD103) has unique structural features (Fig. 7.1) and is the only α-I domain-containing integrin chain that pairs with β7. Beta 7, however, can pair with α4 as well as αE. Both heterodimers are expressed exclusively by leukocytes and have special significance for the mucosal immune system. Alpha 4 beta 7 is the principal mucosal homing receptor for

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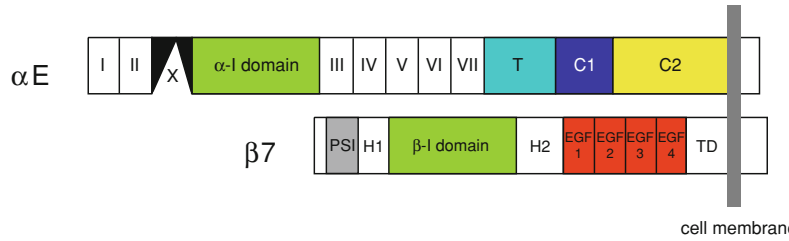

Fig. 7.1 Domain structure of integrin αEβ7. The seven blades of the β-propeller domain of αE are labeled I to VII; the thigh domain, T; the calf-1 and –2 domains, C1 and C2. The extra X-domain, not found in any other integrin chain and containing a post-translational cleavage site, is marked X. In β7, the plexin/semaphorin/integrin domain is labeled PSI; the two components of the hybrid domain, H1 and H2; and the β-tail domain, TD. Note that this figure does not illustrate the relative orientation of the different domains

leukocytes [11] whereas αEβ7 appears to play a role in retention of these cells within or near epithelia. In this review we shall discuss the tissue distribution and induction of αE and present a molecular perspective on the interaction between αEβ7 and its principal ligand, E-cadherin. The complex organization of the αE gene locus will be described. Finally, we aim to present current views of αEβ7 function.

Recent studies indicate that αEβ7 plays an important role in determining the localization of dendritic cell subsets, and therefore indirectly impacts all immune responses, both innate and adaptive. In fact, studies of the αE expressing subset of dendritic cells now dominate the literature on this subject. Of 94 αE integrin references published in 2013, 61 were about αEβ7 expressing dendritic cells. By contrast, in calendar year 2003, there were only 25 references to αE integrin, and none of these were about dendritic cells. The field initially focused on the role of αEβ7 in promoting the functional activities of mucosal T cells, then shifted to a focus to the functional relevance of αE on Tregs then to the current focus on the relevance of αE expression by dendritic cell subsets. The emphasis on the αE expressing subset of dendritic cells is warranted as it is now clear that dendritic cells initiate essentially all immune responses—both innate and adaptive—and thereby play a critical role in defining the nature and character of the immune response. Thus, αEβ7 likely controls these critical processes.

7.2 Tissue and Cellular Distribution

Integrin αEβ7 was originally discovered in the rat, human and mouse by screening panels of monoclonal antibodies for cell surface features that were distinctive for intestinal intraepithelial lymphocytes (IEL) [15, 16, 47]. The original mAbs to αEβ7, RGL-1, HML-1, and M290 (reactive in rat, man and mouse respectively) were subsequently shown to identify a novel integrin alpha chain now known as αE (CD103) [14, 49, 50, 54, 70, 76, 78, 89, 92, 115]. A fourth antibody, MRC-OX62, raised against rat lymphatic dendritic cells was later shown also to recognize αEβ7 [7, 8]. Thus, a distinguishing feature of αEβ7 is that it is expressed most prominently and abundantly in the gut, particularly on T cells in the epithelium [8, 15, 16, 25, 47, 49, 50]. At first, it seemed a foregone conclusion that αEβ7 functioned to retain T cells at mucosal sites, but recent studies reveal a more complex situation. In other compartments of the immune system and among other lymphoid/myeloid cell lineages expression is found on sub-populations which express αEβ7 at lower levels that are, nevertheless, functionally important. In particular, while αEβ7 is expressed by diverse leukocyte subsets, it is now clear that it defines a subset of dendritic cells, and thereby can have a global impact on immune responses.

αEβ7$^+$ T cells are usually found in locations where active TGF-β isoforms are abundant. Expression of αEβ7 on T cells is usually skewed towards the CD8 subset [16, 25, 47], a phenomenon that is readily seen in mixed T cell cultures stimulated with mitogen in the presence of TGF-βisoforms [9, 80, 87]. In the gut, almost all IEL and about half the T lymphocyte population in the lamina propria express αEβ7 [16, 25, 47]. Similarly, the integrin is present on T cells in or near other epithelial surfaces, including those of the lung [80] and genital tract [22, 77]. In lymphoid tissues, including Peyer's patches and mesenteric lymph nodes and in peripheral blood the percentage of αEβ7$^+$ T cells and their level of expression of αEβ7 is generally low [2, 16, 50].

Although αEβ7 was formerly considered to be a mucosal T cell marker, the molecule is also found on other cell lineages. Most studies on the distribution of αEβ7 have failed to detect the molecule on tissue macrophages, but there is an exception in which a proportion of macrophages in lung, liver and lymph node sinuses is reported to have stained positively with mAb HML-1 [100]. An interesting observation was also made that mucosal-type mast cells generated in vitro from bone marrow precursors by culturing in the presence of stem cell factor, IL-3, IL-9 and TGF-β expressed αEβ7 strongly [92, 111]. The presence of the integrin on mucosal mast cells in vivo is strongly supported by circumstantial evidence, but the functional significance and in vivo relevance of such expression remains to be demonstrated.

Significant subsets of dendritic antigen-presenting cells (DC) in the gut mucosa, the mesenteric lymph nodes and the epithelium of the airways of rats and mice are αEβ7$^+$ [7, 8, 46, 65, 73] but in lymph nodes which have no mucosal involvement the proportion is considerably smaller [46] and in the spleen αEβ7 expression is confined to the small subset of CD8$^+$ DC [69]. In man, expression of αEβ7 by mucosal dendritic cells has been less extensively documented but αEβ7$^+$ DC are present in the dome epithelium of Peyer's patches and in the lamina propria [25, 108]. In contrast, Langerhans-type DC generated in vitro from hematopoietic stem cells in the presence of TGF-β and other cytokines do not express the integrin [79].

Detailed scrutiny of B cell subsets for expression of αEβ7 has revealed a complex picture. Early studies showed that the integrin was expressed by few if any B cells in the gut mucosa or elsewhere. However, Csencsits et al. [23] identified a population of αEβ7$^+$ B220$^+$ cells in the intestinal mucosa following intranasal immunisation of mice with cholera toxin. That cells of B lymphocyte lineage can, in certain circumstances, express αEβ7 is supported by the detection of a small population CD19$^+$ αE$^+$ B cells in peripheral blood [40] and also by much earlier observations that αEβ7 expression is a diagnostic marker for hairy cell leukemias [70–72].

Studies of αEβ7 expression during thymic ontogeny in the mouse have shown that 3–5 % of cells express the integrin and that it is represented in both TCRαβ and γδ lineages, particularly in the late developmental stages [2, 59]. The integrin is present on about half the population of thymic precursors of dendritic epidermal T cells (DETC) and on all mature cells of this subset [59]. In humans, αEβ7 was found to be expressed by a major subpopulation of single positive CD8$^+$ human thymocytes and a smaller proportion of less mature double negative cells [56, 67]. Recent studies implicate Runx 3 in controlling αEβ7 expression during thymocyte development [33, 112], and indicate that CXCR3 and αEβ7 both are expressed by the CD8$^+$ single positive thymocyte subset [4], and that Treg likely derive from Foxp3$^+$ double positive (CD8$^+$CD4$^+$) cells that lack αEβ7 expression [75]. It has also been reported that most, if not all, naïve CD8$^+$ that have recently emigrated from the thymus into the circulation express αEβ7 [67]. Thus, the frequency of αEβ7$^+$ CD8 T cells in the blood with a naïve phenotype appears to be a useful indicator of thymopoiesis. Maintenance of αEβ7 expression by this cell population, and also by splenic and blood CD8$^+$ T cells, has been reported to depend on lymphotoxin alpha (LTα) [31]. However, the possibility was not excluded that LTα

induces expression of the αE subunit by an indirect effect on TGF-β processing. Single positive thymocytes expressing αEβ7 may migrate to the small intestine via a sphingosine 1-phosphate (S1P) dependent process [55].

The study of T regulatory (Treg) cells (formerly known as suppressor T cells) has undergone a renaissance and their importance in immune homeostasis and in the prevention of autoimmune diseases and allograft rejection is clear. In vivo models of suppression of autoimmunity involving adoptive cell transfer and in vitro studies on suppression of lymphocyte proliferation by spleen or lymph node T cell subpopulations have shown that Treg cells reside within a population that is $CD4^+$ $CD25^+$ $CD45RB^{low}$ [17, 64]. Four studies have shown that αEβ7 is expressed by 20–30 % of this T cell subset [32, 60, 68, 117]. Similarly, regulatory $CD8^+$ T cells generated by co-culture of intestinal epithelial cells and peripheral T cells were shown to express αEβ7 [1].

7.3 Induction of αEβ7

It is long been recognized that transcription of the αE subunit is regulated by transforming growth factor beta (TGF-β) [49, 50, 76, 85, 92]. Such induction is commonly attributed to the TGF-β1 isoform but all isoforms of TGF-β (also mouse TGF-β2, and -β3 for example) exhibit this property (GAH unpublished data); it has not yet been established which of the TGF-β isoforms contribute to αEβ7 induction in vivo. Recent studies point to a key role for membrane bound TGF-β in this process [113, 114], but a complete understanding of this important interaction is muddled by our poor understanding of how TGF-β isoforms are processed to their active forms in the particular cells used in these experiments. It has been reported that ligation of β1 integrins can act synergistically with TGF-β in αE induction [80], and that activation of naïve human $CD8^+$ T cells with anti-CD3 in the presence of IL-4 can also increase αEβ7 expression [99], though it is unclear if these apparent inducers operate through the indirect action of TGF-β isoforms.

It is widely held that αEβ7 expressed by T cells located in the vicinity of epithelia is induced locally by TGF-β isoforms produced mainly by epithelial cells. This view is supported by the observation that T cells stimulated in vitro by co-culture with allogeneic kidney epithelial cells, or T cells that migrate into epithelial monolayers, are induced to express αEβ7 and that expression is blocked by anti-TGF-β antibody [34, 90]. The results of a study of mucosal T cell memory by Kim et al. [51] are also consistent with the idea that αEβ7 is upregulated locally. Ovalbumin-specific transgenic $CD8^+$ T cells were adoptively transferred to recipients that were then infected with recombinant vesicular stomatitis virus expressing ovalbumin (VSV-OVA). Analysis of donor-type memory cells in various lymphoid compartments indicated that αEβ7 was strongly upregulated on IEL over the 5 week study period.

The notion that αEβ7 expression is mainly, if not solely, TGF-β-dependent is supported by a study showing that in transgenic mice which express the negative regulator of TGF-β isoform signalling, Smad7, under an *Lck* promoter, 50 % of intraepithelial T cells in the gut no longer express αE [96]. Expression of the integrin by the remaining cells probably reflects insufficient expression of the transgene in this population but leaves open the possibility that an alternative signaling pathway could be responsible for αE expression in these circumstances. Using a T cell line, Robinson et al. showed that TGF-β induces αE transcription de novo within 30 min [85]. The speed of induction suggests that synthesis of signaling intermediaries or new transcription factors was probably not required. These authors also looked for transcription control elements in the promoter region of the human αE gene using deletion analysis to examine 4 kb of genomic sequence upstream of the transcription start site. Although the promoter functioned well in reporter assays, it bestowed neither cell lineage specificity nor TGF-β responsiveness. Thus, transcription control mechanisms for αE are

likely to be considerably more complex than those of most other integrin α-chain genes, whereas lineage specificity is determined by the proximal promoter in other integrins.

7.4 Gene Structure

Past studies established the complexity at the locus of the integrin αE gene, *Itgae*. Schön et al. [88] generated a partial map of murine *Itgae*, and subsequently the human genome sequencing project provided more complete information on human *Itgae* [37]. Human *Itgae* contains 31 exons spanning approximately 85 kb (Fig. 7.2). Comparison with the genes encoding the closely related αM and αX integrin proteins [21, 27, 74] reveals a highly conserved gene structure. All the introns are located in similar positions and have the same phase in the three genes, although *Itgae* contains an extra exon (exon 6) that encodes the X domain not present in other integrins (see Fig. 7.1). The α-I domain is encoded by exons 7–10. Human *Itgae* is found at chromosome 17p13.3 rather than in the αL/αM/αX/αD integrin cluster at chromosome 16p11 [21, 110], and is syntenic with that of murine *Itgae* on chromosome 11 [88]. Robinson et al. [85] analyzed the transcription start site of human *Itgae*, and identified two start sites 51 and 44 bp upstream of the start codon, and a third possible initiation site around position ~90 bp. Interestingly, another gene, *Gsg2*, that encodes the mitotic protein kinase Haspin is found on the opposite strand within an intron of *Itgae* Fig. 7.2. The Haspin promoter appears also to drive expression of a truncated and alternatively spliced *Itgae* transcript that is widely expressed and could function as a non-coding RNA [37].

The human β7 gene, *Itgb7*, comprises 14 exons spanning approximately 10 kb and maps to chromosome 12q13.13 [5, 42], syntenic with murine β7 on chromosome 15 [116]. The intron-exon structure of *Itgb7* is more similar to that of the β1 and β2 genes than the β3, β5 and β6 genes, consistent with a similar sub-grouping derived from analyses of sequence homology [42].

7.5 Ligand Binding

Expression of αEβ7 by T cells closely juxtaposed to epithelial surfaces suggested that this integrin might bind a counter-receptor on the surface of epithelial cells. In 1993 three groups reported that a ligand for αEβ7 was present on epithelial cell lines [12, 81, 82]. The epithelial ligand was later identified as the homophilic adhesion molecule E-cadherin [13, 39, 44], and mutagenesis studies combined with crystal structure determination and molecular modeling led to a detailed model for αEβ7 binding to E-cadherin in which the MIDAS motif within the α-I domain of αE makes direct contact with an acidic residue at the tip of domain 1 in E-cadherin (Fig. 7.3) [38, 41, 45, 98]. These findings strengthened the concept that αEβ7 retains leukocytes in epithelial tissues by binding to E-cadherin on epithelial cells.

E-cadherin expression is found on most epithelial cells, but is not limited to this population. Recent studies suggest that E-cadherin can act at the level of dendritic cells to impact immune responses. For example, Siddequi et al. [91] observed that monocyte-derived inflammatory DCs express E-cadherin, and that these promote intestinal inflammation. Similarly, Uchida et al. [102] reported that E-cadherin and αEβ7 on DETC regulate their activation threshold through binding to E-cadherin on keratinocytes. Van den Bossch et al. [104, 105] detailed the regulation and function of the E-cadherin/catenin complex in cells of the monocyte-macrophage lineage and DCs, and found that E-cadherin is expressed by alternatively activated macrophages. Thus, αEβ7 expressing cells potentially interact with and regulate diverse leukocyte populations, but the extent to which this occurs in vivo has yet to be established.

E-cadherin is the only well-defined counter-receptor of αEβ7, but there is preliminary evidence for at least one further ligand on keratinocyte cell lines and intestinal lamina propria endothelial cells that lack E-cadherin expression [10, 41, 93].

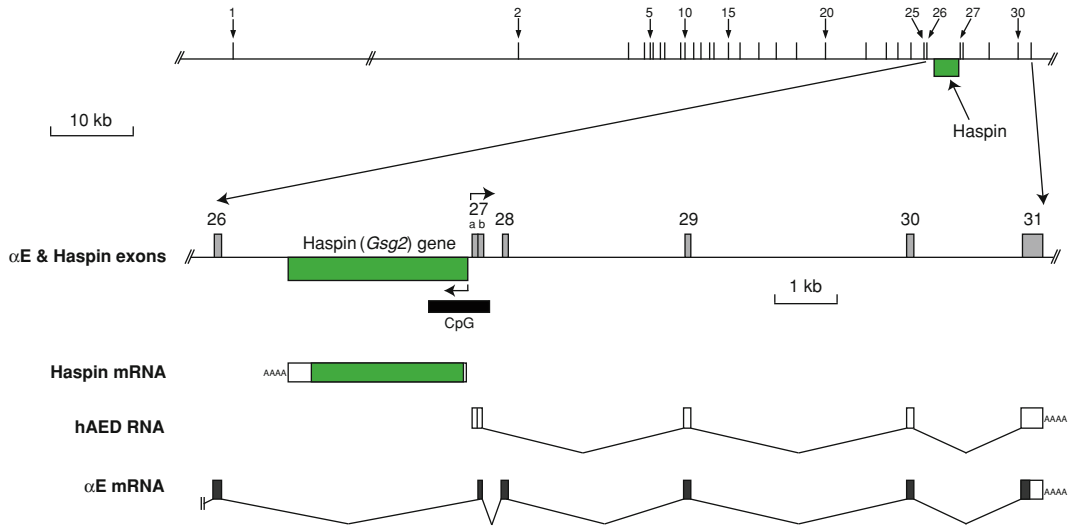

Fig. 7.2 Outline structure of the αE genomic locus. The *top line* shows the intron-exon structure of the integrin αE (*Itgae*) and Haspin (*Gsg2*) genes. On the *second line*, the 3′ region of the αE gene containing the Haspin gene is shown in more detail. Intronic regions are shown as *horizontal lines* and exons as *boxes*. *Bent arrows* represent transcription start sites, and the *thick black line* indicates the location of a CpG island. The *three lower lines* show the three transcribed products of this genomic region, including the Alpha-E derived mRNA, hAED. In each case, *boxes* represent exonic portions of RNA, *thin lines* indicate introns, *dark shading* indicates protein coding regions, and AAAA indicates a poly(A) tail

7.6 Function

7.6.1 Effector and Memory T Cells

It is now clear that αE controls the accumulation of effector and memory T cells (resident memory T cells, T_{rm}) in non-lymphoid tissues and thereby may promote their capacity to eliminate invading pathogens. Alpha-E expression marks T_{rm} cells in a variety of tissues [63, 107] (25), and there is good evidence that such expression promotes their local persistence, particularly for intraepithelial $CD8^+$ T cells in the intestinal and vaginal mucosa, where binding to E-cadherin may be critical [88, 107]. Moreover, it is not clear that αEβ7 expressing T cells present at all sites are exclusively memory T cells, in that many are present in naïve mice prior to specific antigen exposure. The underlying mechanisms regulating αEβ7 expression by T_{rm} remain poorly defined but are likely similar to those described above for other αEβ7 expressing cells. These include induction of αEβ7 and downregulation of the chemokine receptor CCR7 with a dominant role for local TGF-β activity in the process. Suvas et al. have shown that systemic and mucosal infection both are effective in generating mucosal $\alpha E\beta 7^+$ T_{rm} responses [95]. Yu et al. [113] have also reported that human $CD1c^+$ DCs express cell surface TGF-β and thereby drive the generation of αEβ7 expressing cytotoxic lymphocytes (CTL).

7.6.2 Allografts

A number of studies have examined whether αEβ7 on T cells could play a role in allograft rejection. Hadley et al. [35, 36] reported that up to 63 % of T cells infiltrating renal allografts undergoing a late rejection crisis expressed αEβ7 and that the cells were localized mainly in the tubular epithelium. Similar findings were reported by Robertson et al. [83, 84] who observed a correlation between the prevalence of

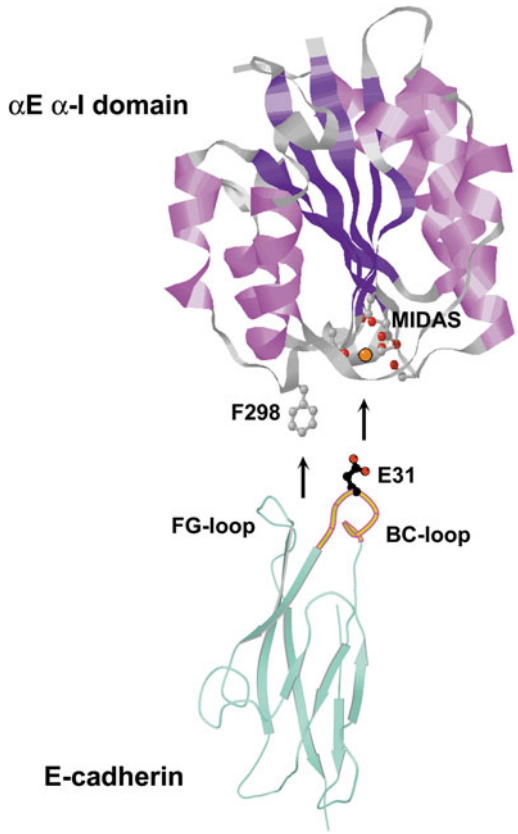

Fig. 7.3 A model of the αEβ7 integrin α-I domain docked onto E-cadherin domain 1. Residue E31 in the BC-loop of E-cadherin is predicted to coordinate the MIDAS magnesium ion in the α-I domain and F298 of αE is predicted to project into a hydrophobic pocket between the BC and FG loops of E-cadherin. For details see ref [38]. Reproduced with permission from Agace WW, Higgins JMG, Sadasivan B, Brenner MB, Parker CM. Curr. Opin. Cell Biol. 2000; 12:563–568 (Copyright 2000, Elsevier Science)

αEβ7$^+$ cells in the tubular epithelium, the severity of tubulitis and the levels of TGF-β in the epithelium. Earlier studies established that αEβ7 was induced on CD8$^+$ T cells co-cultured with renal epithelial cells [34] and that αEβ7 provided accessory function for cytotoxic lysis of target epithelial cells [86]. This evidence supports the view that αEβ7 is induced on infiltrating CD8$^+$ cells by TGF-β produced locally in the allograft and causes the cells to accumulate in the graft epithelium by adhesion to E-cadherin expressed by tubular epithelial cells. The integrin/ligand interaction would then provide accessory function for cytotoxic lysis or cytokine production. This interaction may be especially important in rejection when other integrin/ligand interactions, principally αLβ2 (LFA-1)/ICAM-1, are unavailable. This view is strongly supported by the observation that αE null/null mice are unable to reject pancreatic islet allografts [26, 48]. Although CD8$^+$ cells accumulate around the graft they do not come into intimate contact with islet cells, which are known to express E-cadherin but not ICAM-1. This view is further supported by the observation that T cells from αE null/null mice do not elicit gut graft-versus-host disease (GVHD) on transfer to wildtype allogenic recipients [24]. Zhou et al. [118] confirmed these findings in a rat GVHD model, and further observed that the skin epidermis in rats during GVHD is infiltrated by an equal number of CD4$^+$ T cells and CD8$^+$ T cells expressing αEβ7. Collazo et al. [19] reported that expression of SH2 domain–containing inositol 5-phosphatase (SHIP) is required for robust expansion of donor αEβ7$^+$ CD4$^+$ T cells during graft-versus-host and host-versus-graft responses by CD4$^+$ T cell and limits their immunoregulatory capacity. These observations on the role of αEβ7 in allograft rejection and GVHD identify a potential opportunity for therapeutic intervention using inhibitors specific for this integrin.

Separation of deleterious GVHD pathology from beneficial graft-versus-leukemia (GVL) responses following bone marrow transplantation (BMT) remains a major challenge in the treatment of hematologic malignancies by allogeneic hematopoietic cell transplantation (HCT). Liu et al. [62] used αE null/null mice to show that αEβ7 expression by CD8$^+$ T cells is required for the former but not the latter process, identifying αEβ7 blockade as an improved strategy for GVHD prophylaxis. Li et al. [61] showed that preconditioning of host mice with anti-CD3 mAb also separates GVHD and GVL effects, and does so by reducing the number of αEβ7 expressing dendritic cells in the mesenteric lymph nodes.

7.6.3 Tumor Immunology

Le Floc'h et al. [29, 57, 58] reported that αEβ7 expression by $CD8^+$ CTL clones in tumors can be induced by TGF-β expression within the tumor. These studies also showed that αEβ7 can participate in formation of the immunological synapse between the CTL and the tumor target, and that interaction with E-cadherin expressed by the tumor target is required for polarization and subsequent release of cytotoxic granules. Subsequent studies showed that interaction of αEβ7 with E-cadherin, but not αLβ2 with ICAM-1, acts at the level of the immunologic synapse formed between tumor-infiltrating lymphocytes and tumor cells to promote CCR5-dependent retention of CTL [30], that interaction of αEβ7 with E-cadherin promotes the phosphorylation of the ERK1/2 kinases and Phospholipase C-γ1 (PLC-γ1), which is sufficient to induce the polarization of cytolytic granules [57], and that interaction between CTL and epithelial tumor cells is regulated by αE expression at the immune synapse which can profoundly influence effector functions of CD8 T cells [29]. Thus, αEβ7 potentially plays a role in tumor elimination through interaction with E-cadherin. These findings raise the exciting possibility that the characteristic loss of E-cadherin expression and gain in invasiveness by metastatic epithelial tumors exhibited by many neoplastic epithelial cells [97] might, in part, reflect CTL selection. That said, the frequency of tumor-reactive CTL clones that express αEβ7 remains a matter of speculation. Nonetheless, together, these studies provide novel insight onto the role of αEβ7 in CTL function. Also, of relevance to the field of tumor immunotherapy, Trinite et al. [101] reported that immature ($CD4^-$ αE$β7^+$) rat dendritic cells can induce rapid caspase-independent apoptosis-like cell death and subsequent phagocytosis of tumor targets. Both of these sets of findings have spurred interest in the development of novel immunotherapeutic strategies to combat cancer.

7.6.4 T Regulatory Cells

The role of αEβ7 in Treg function is controversial and highly dependent on the model employed. However, there is evidence that αEβ7 plays an important role in promoting both the function and localization of Treg cells, and even that αEβ7 marks Tregs with the most potent immunosuppressive properties. McHugh et al. [68] and Lehmann et al. [60] both reported that that the αE$β7^+$ population was more efficient at suppressing anti-CD3 stimulated proliferation of $CD4^+$ CD25-cells than the αEβ7-subset. TGF-β plays a role in the development and function of Treg cells, and the presence of αEβ7 on the surface of a major subpopulation of Treg cells argues, at least, that these cells have recently been exposed to TGF-β. However, such expression may be misleading and it remains to be determined if a direct role of αEβ7 on Treg is always relevant. As described below, αEβ7 expressing dendritic cell subsets can also control the suppressive function of Tregs.

More recently, Suffia et al. [94] have shown that αEβ7 plays an essential role in retention of Treg and control of Leishmania major infection, and that targeted disruption of the αE gene renders mice susceptible to Leishmania infection, a result that could be reversed by transfer of αEβ7 expressing Tregs from wild type mice. In contrast, the Powrie group has reported that targeted disruption of αE has no effect on the suppressive capacity of Tregs in a mouse model of colitis [3]. Rather, expression of αEβ7 by dendritic cells was found to be necessary for Treg function (see below). Van et al. [106] showed that CD47 controls the in vivo proliferation and homeostasis of the αEβ7 expressing subset of peripheral Tregs. There is also evidence that αEβ7 expressing CD8 T cells can be suppressive. For example, Uss et al. provided evidence that αE$β7^+$ CD8 T cells can be potently immunosuppressive in vitro [103], effectively functioning as T regs.

7.6.5 Dendritic Cells

While the precise function of each dendritic cell (DC) subset remains to be clearly defined, it is clear that expression of αEβ7 allows DCs to control the balance of effector responses to foreign antigens. Annaker et al. reported that αEβ7 expression by DCs is required for the induction of Tregs to suppress intestinal bowel disease [3]. In this model, αEβ7$^-$ DCs promoted mainly effector cytokine IFN-γ production by the responding T cells whereas αEβ7$^+$ DCs enhanced immune protection by inducing the gut homing receptor CCR9 on responding T cells. These data indicated that αEβ7 can control the balance of effector vs regulatory T cell activity in the intestine. Indeed, Coombes et al. have shown that mucosal αEβ7$^+$ DC induce Foxp3$^+$ Treg by a TGF-β and retinoic acid-dependent pathway [20]. Subsequent studies confirmed that retinoic acid is centrally involved in regulating this pathway [53], and that human αEβ7$^+$ DC share this ability to induce T reg [108]. Choi et al. [18] reported that DC are dominant in normal aortic intima and, in contrast to macrophages which promote atherosclerosis, the αEβ7$^+$ DC subset was associated with protection from atherosclerosis. Weiner et al. reviewed the existing literature on oral tolerance and also concluded that αEβ7$^+$ DCs induce T regs [109].

There is also evidence that αEβ7$^+$ DC subsets can indirectly *promote* immune responses. For example, αEβ7$^+$ DC appear adept at generating gut-tropic effector CD8 T cells [43]. Recent studies provide further insight into the antigen-presenting qualities of αEβ7$^+$ dendritic cells. Bedoui et al. [6] reported that αEβ7$^+$ DCs in non-lymphoid tissues are specialized in the cross-presentation of cell-associated antigens and are essential for inducing proliferation of CD8 T cells, a finding that appears consistent with recent work in human DC [108].

7.6.6 Innate Immune Responses

McCarty et al. [66] reported that circulating Vδ2 T cells display enhanced gut-homing potential upon microbial activation and populate the human intestinal mucosa, generating functionally distinct αEβ7$^+$ and αEβ7$^-$ subsets that promoted inflammation by colonic αβ T cells. Further evidence that αEβ7 functions in innate immune responses is provided by the findings of Kinnebrew et al. [52] who reported that αEβ7$^+$ CD11b (αMβ2)$^+$ DCs in the lamina propria, in addition to promoting long-term tolerance to ingested antigens, also rapidly produce IL-23 in response to detection of flagellin in the lamina propria. Flores-Langarica et al. [28] showed that systemic flagellin immunization can induce mucosal immune responses.

7.7 Conclusions

Integrin αEβ7 has proved to be enigmatic and tantalising. Considerable efforts to define its true significance in vivo have met with mixed fortunes. Whilst this integrin undoubtedly contributes to mucosal specific retention of diverse leukocyte subpopulations there are valid grounds in the future to seek deeper significance in its signaling capacity, especially in relation to cross-talk with the epithelium. Studies of αE knockout mice have clearly identified an important role for this integrin in allograft rejection and also have provided a glimpse of its possible significance in immunoregulation. Resonance with the finding that αEβ7 is expressed by major leukocyte subsets is striking and the functional relationships between these observations provide fertile ground for further investigation. It is evident, for example, that while αEβ7 expressing mouse dendritic cells are important, the molecular function of αEβ7 in this context, and on T_{rm} in the brain, is less clear. The role of αEβ7 on similar cells in humans also invites further study. TGF-β signaling to both the αEβ7 expressing leukocyte and its target (if any), and the significance of cell surface-bound TGF-β, merit further attention. In mice, the αE integrin gene locus is sandwiched between the Th2 cytokine gene cluster (IL-4, IL-5 and IL-13) and a cluster of chemokine genes (eotaxin, TCA-3, MCP-1, 3, 5, MIP-1α and 1β, RANTES). In future studies to address the role of αEβ7 in immunoregulation it will be essential to

utilize αE null/null and control mice that are congenic at the Th2 and chemokine loci, and to use conditional knockout mice with disruption of the gene targeted to specific leukocyte subsets.

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Structural Aspects of Integrins

8

Robert C. Liddington

Abstract

Structural studies on integrins have recently made great strides in recent years. Crystal structures of the complete extracellular fragments of three integrins in open and closed conformations, 6 α-I domains in complex with ligands, and at least 20 intracellular proteins in complex with cytosolic tails have been obtained; and several transmembrane and cytosolic complexes have been determined by NMR. High resolution EM studies complement these atomic resolution techniques by studying the integrin in different activation states. Although we still have only a few experimental examples among integrin family members, the high level of sequence homology between integrins means that reliable models can be built for the other members of the integrin family. These structures make sense of a lot of preceding biochemical, biophysical and mutagenesis studies, and generate many new testable hypotheses of integrin function. This chapter emphasizes new structural insights applicable to all integrins, with an emphasis on those integrins that contain an α-I domain. The structural data reinforce the notion of the integrin as a molecule in dynamic equilibrium at the cell surface, regulated by binding both to extracellular and intracellular ligands.

Keywords

Integrins · Structure · Mechanism · Allostery

8.1 Overall Structure

Integrins are $\alpha\beta$ heterodimers, consisting of a head domain from which emerge two legs, one from each subunit, ending in a pair of single-pass transmembrane helices and short cytoplasmic tails,

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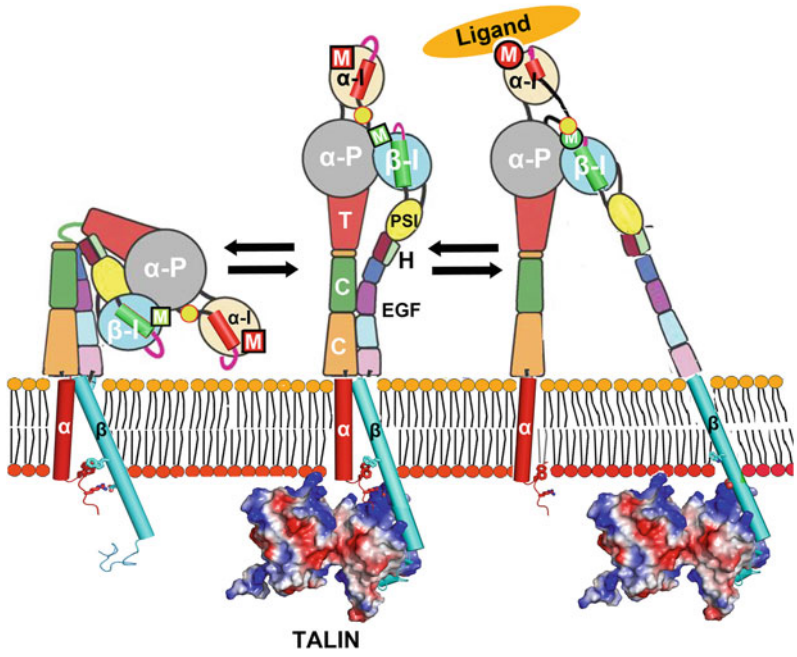

Fig. 8.1 Cartoon of the αXβ2 structure derived by crystallography and EM studies. At *left*, the bent, low affinity integrin stabilized by bonds between the head, legs and cytoplasmic tails. At *center*, an unknown trigger causes the integrin to "stand up", while maintaining most of its low affinity bonds. At *right*, binding of activated talin and/or binding of an extracellular ligand, trigger an open, high affinity form of the integrin, with TM helices separated

except for α6β4 (Fig. 8.1). The integrin "head" comprises a seven-bladed propeller from the α-subunit that makes an intimate contact with the β-I domain. Nine α-subunits (α1, α2, αl0, α11, αD, αE, αL, αM and αX) contain an additional domain, the α-I domain, that is inserted between two loops on the upper surface of the propeller, where it plays a central role in ligand binding [27, 41, 58]. The α-I domain contains an invariant ligand binding site called MIDAS, for Metal Ion-Dependent Adhesion Site [34], in which a metal ion is coordinated by three loops from the I domain, and a glutamic or aspartic acid from the ligand completes an octahedral coordination sphere around the metal. In those integrins that lack an α-I domain, the β-I domain and propeller form the major ligand recognition sites; in the α-I domain integrins, the β-I domain plays a regulatory role.

In the absence of ligand, bonds between the legs, tails and head are believed to hold the head in an "inactive" or "resting" conformation that has low affinity for ligand [20, 56, 61]. Recent structural data suggest that integrins possess three global conformations (see Fig. 8.1): a bent conformation in which the head adopts a "closed", low affinity conformation and the cytoplasmic tails form an inhibitory complex; an extended conformation of the head that retains its low ligand affinity; and a high affinity form in which the legs and tails separate, and the "hybrid" domain, which is part of the head, swings away from the β-I domain, propeller and α-I domain, promoting conformational changes that create high affinity binding sites on both the head and tail [54]. During "outside-in" signaling, the head binds to ECM proteins or counter-receptors on other cells, triggering conformational changes that propagate down the "legs" and through the plasma membrane, leading to a reorganization of the C-terminal tails that allows them to bind intracellular proteins [46]. During "inside-out" signaling, cytosolic proteins bind and sequester one or both of the cytoplasmic tails, triggering conformational changes in the head that promote a high affinity "active" integrin [19, 60], in which the integrin "stands up".

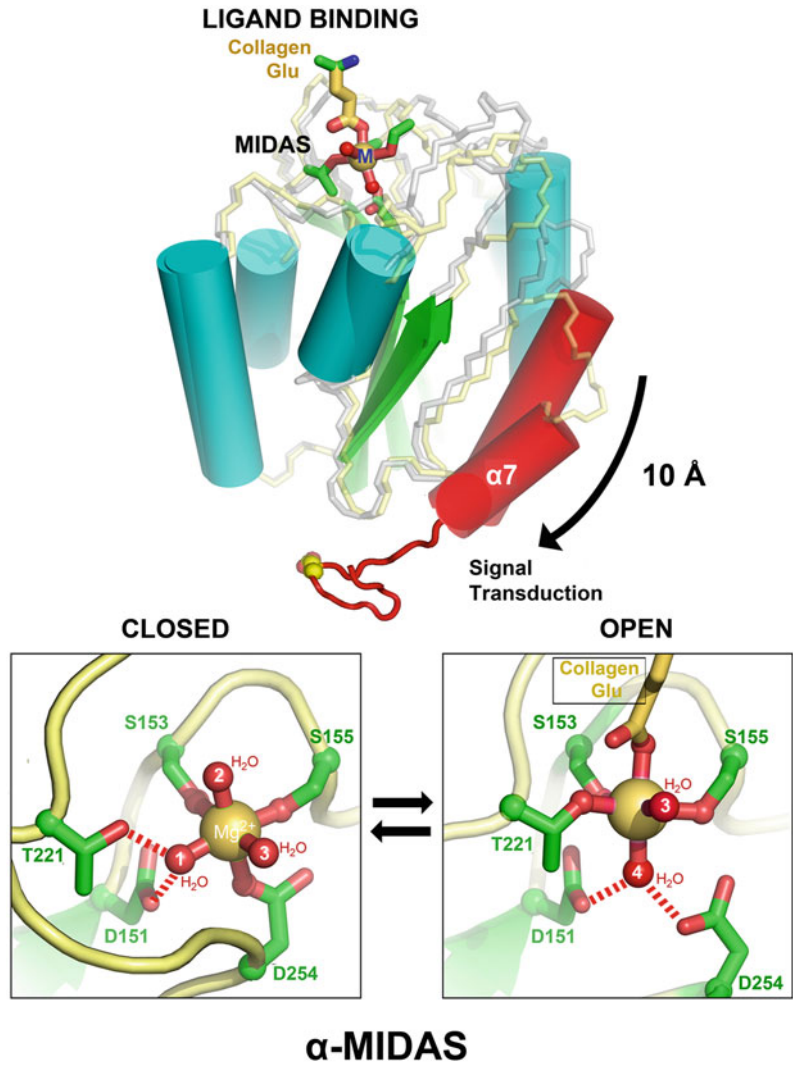

Fig. 8.2 Two conformations of the α-I domain. *Upper panel* conformational changes in the α2-I domain on binding collagen. *Lower panel* Conformational changes in the α-MIDAS motif upon binding ligand. At *left*, the "closed" conformation observed in the absence of ligand; at *right*, the "open" conformation seen when ligand is bound. These changes are mechanically linked to the tertiary changes in the domain. It is very likely that all α-I domains undergo the same switch

8.2 The α-I Domain

The first crystal structure of an α-I domain revealed a compact domain comprising a central mostly parallel β-sheet surrounded on both sides by amphipathic α-helices [34] (Fig. 8.2). Subsequent crystal structures of recombinant αL, α1 and α2 I domains display the same three-dimensional fold, as expected given their reasonable sequence similarity [15, 43, 45]. The MIDAS motif lies at the C-terminal end of the central β-sheet, with three loops contributing sidechains that coordinate the metal ion (Fig. 8.2 Lower panel). The metal-coordinating MIDAS residues are invariant among α-I domains, and mutagenesis of any of these residues abrogates ligand binding. Surface-exposed sidechains surrounding the MIDAS motif are more variable; they provide additional ligand contact points and hence ligand specificity [26, 41, 52].

The structures of 6 ligand-bound α-I domains have now been determined. The first was the α2-I domain bound to a collagen-like triple helix [17]. More recently, the structures of the αL-I domain in complex with homologous fragments

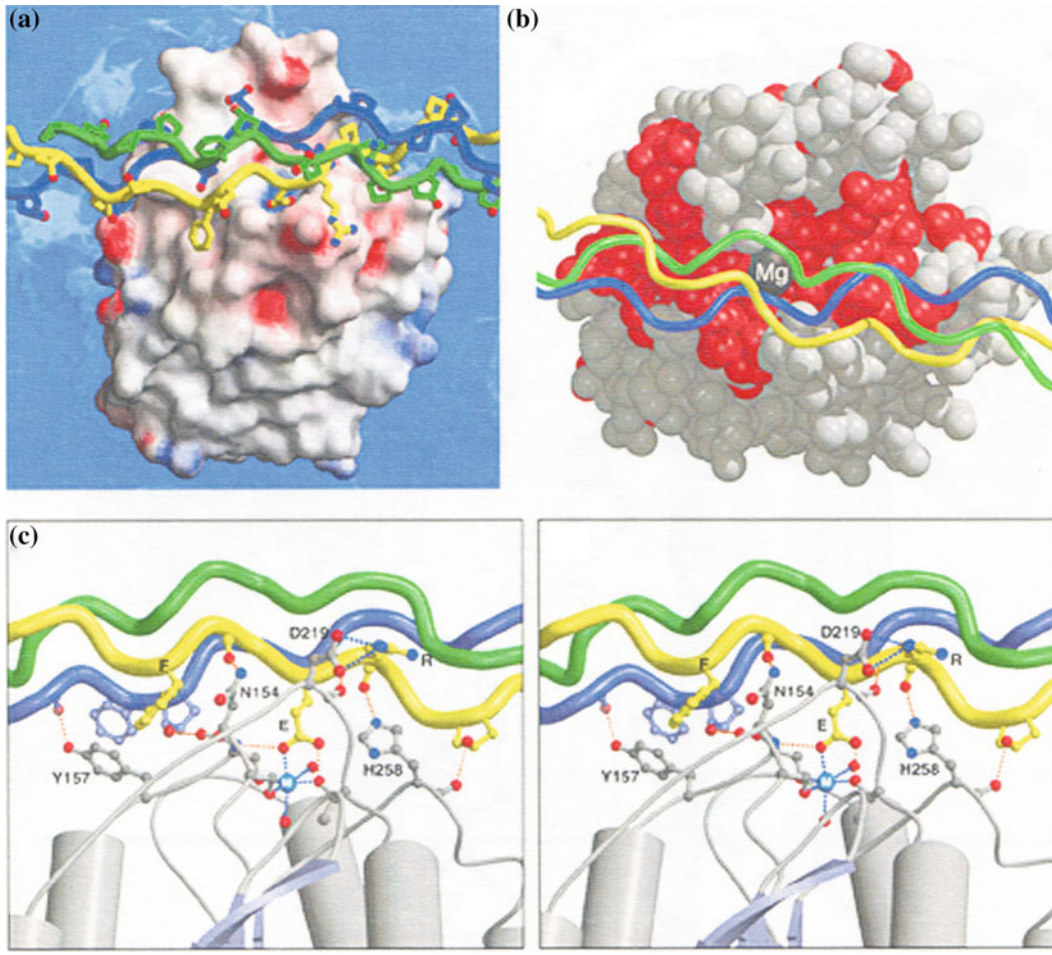

Fig. 8.3 Collagen binding to the α2-I domain. **a** Surface model of the α2-I domain colored by surface charge (*red* = negative, *blue* = positive) with a triple helical fragment of collagen bound. **b** Space-filling model of the complex (rotated about a horizontal axis compared with **a**), showing residues (in *red*) that are invariant in the collagen-binding integrins, α1β1, α2β1 and α10β1. The strong conservation of the binding surface suggests that these integrins will engage collagen in the same fashion. **c** Stereo close-up image of the α2-I:collagen complex

of ICAM-1 [51], ICAM-3 [53] and ICAM-5 [67] have been determined. Recently, the first authentic complex of an αM-I domain bound to ligand (the C3d domain of complement C3) [2] validates the earlier structure of the αM I domain bound to a "ligand-mimetic" crystal contact [33, 34]. They all demonstrate that ligand binding triggers a profound conformational switch in the α-I domain that underlies affinity regulation and signal transduction. The conformational switch is essentially identical in all these examples, strongly suggesting that all α-I domains will undergo the same switch.

8.3 Structural Determinants of Collagen Binding

Recombinant α2-I domain was crystallized as a complex with a homotrimer of a 21-mer peptide containing a critical GFOGER (where O is hydroxyproline) motif [17, 30]. The peptide closely resembles the structure of uncomplexed collagen-like peptides [16], and has the properties of a folded protein domain (i.e., stable secondary and tertiary structure). Three loops on the upper surface of the α2-I domain that comprise

the MIDAS motif also engage the collagen, with a collagen glutamate completing the coordination sphere of the metal (Fig. 8.3). The critical roles of both the MIDAS and surrounding residues have been confirmed by mutagenesis [52].

The buried surface area on complex formation ($\sim$1,200 A^2) is at the lower limit of known protein-protein interfaces (the value is almost identical for the αL-I:ICAM complex), especially given the fact that some of the binding energy must be expended in switching the conformation of the I domain from closed to open. The quite reasonable affinity of the interaction (Kd = 35–90 nM) [24] reflects the unusually strong bonds formed by the glutamate-metal-I domain bridge, which has been estimated to contribute $\sim$5 kcal/mol. This bridge is indeed critical, since the conservative substitution of collagen Glu to Asp in the GFOGER motif eliminates binding [31], presumably because the aspartic acid is too short to reach down from the rigid collagen triple helix to bind to the partly buried metal ion. However, in the case of the αM-C3d interaction, Asp is the preferred residue, perhaps because it lies on a flexible loop at the end of a helical segment [2].

The MIDAS motif and much of the collagen-binding surface are strictly invariant among the collagen-binding I domain integrins (αl, α2, α10 and α11), suggesting that these integrins will all engage collagen in a similar fashion, with a strict requirement for glutamate in the collagen motif. The periphery of the binding surface is more variable, however (Fig. 8.3b), which would explain their collagen type preferences. The recent structure of the α1-I domain bound to collagen containing the closely related motif, GLOGEN, confirms this [11]. In addition, a gain-of-function point mutation in the α2-I domain (i.e. one that favors the open conformation) [10] displays relaxed specificity and alternate binding modes to the GFOGER motif. Given the special nature of collagen (see Chap. 3 by Zutter and Santoro), this observation may point to profound consequences for collagen recognition by activated cells.

8.4 The Integrin α-I Domain and the von Willebrand Factor (vWF) A Domain: A Caveat

The integrin α-I domain is generally categorized as a member of the vWF-A domain superfamily, based on sequence similarity and a highly conserved overall 3-dimensional structure. However, since MIDAS- and non-MIDAS containing vWFA-domains have distinct ligand-binding and allosteric properties, this author believes that much confusion could be avoided if the family was reclassified into two sub-families: Two examples illustrate my point. First, the eponymous vWF-A1 and vWF-A3 domains lack at least one of the acidic residues of authentic MIDAS motifs, and so do not bind metal; moreover, they bind ligands via distinct surfaces, and conformational changes are not induced [21]. In fact, vWF-A3, like integrin α2β1, binds triple-helical collagen, but it utilizes a different surface (one side of its β-sheet) [7]. Second, the "vWF-A domain" of Factor B *does* contain a functional MIDAS motif, and binds an acidic moiety in its ligand, complement iC3b, in the canonical integrin fashion; in this case, the metal ion engages the C-terminal carboxylate iC3b, triggering integrin-like conformational changes [18], suggesting that it should be classed as an I domain. Indeed, a genome-wide collection of vWF-A domains has been compiled [62] and have been these subdivided into I-like and A-like domains based on the conservation of key MIDAS residues.

8.5 Conformational Changes in the α-I Domain

Ligand binding alters the conformation of α-I domains in the same way in the three cases studied thus far (α2, αM and αL), as well as in a subset of "vWF-A domain" (as noted above) and the matrix receptor, TEM8 (in complex with pathogen; see below). Binding of an acidic residue to the α-MIDAS causes a switch in in Mg^{2+}

coordination in which a direct bond to a MIDAS threonine is gained while a direct bond to an aspartic acid is lost (Fig. 8.2b, c). These subtle changes in metal coordination are linked to extensive secondary and tertiary changes that create a complementary surface for binding ligand and generate a 10 Å downward movement of the C-terminal helix, α7. The helix movement links the change in the upper ligand-binding surface to the lower surface of the domain. The shift of the helix α7 is highly significant in the context of the whole integrin, since the helix is packed against the propeller and β-I domain (see Sect. 8.8).

The close similarity between the structural changes seen in the three α-I domains and a subset of A domains suggests that there are just two principal conformations for I domains, "open" and "closed" (Fig. 8.2). The "open" conformation is seen in the presence of ligand or ligand mimetic, while the "closed" conformation is seen in the absence of ligand. It therefore appears to be the formation of a strong ligand-metal bond, requiring a change in metal coordination, that triggers the conformational switch. Springer's group has engineered disulfide-linked αL-I domains with intermediate affinity and packing of the C-terminal helix, and suggested the existence of an intermediate state [51]. However, in these structures the MIDAS motif exists in only two conformations, and it remains to be seen whether the intermediate conformation has biological relevance or is an artifact of the engineered disulfide. It should be noted that it is not necessary to invoke an intermediate tertiary conformation in order to explain an intermediate affinity. In principle, a shift in the position of the equilibrium between two states is sufficient [38].

Various studies have now been published in support of the hypothesis that the open and closed conformations of the α-I domain equate with high and low affinity states. Thus, mutants of the αM-I domain that are predicted to destabilize the closed conformation and favor the open conformation increase the affinity for the ligand iC3b [41]. The epitope for an antibody that binds only to the high affinity form of the αMβ2 integrin maps to a region that undergoes extensive conformational changes between the closed and open forms [35].

Disulfide engineering studies on recombinant I domains and full-length integrins, which lock the domain either into the open or closed state, also support the hypothesis [39, 49, 50]. So does the structure of the αL-I domain in complex with the inhibitor lovastatin [25], which reveals allosteric inhibition by binding between the β-sheet and the C-terminal helix, preventing the helical shift.

It should also be appreciated that pathogens often utilize integrin α-I domains for cell entry, and there is evidence that many bind across the MIDAS motif. However, in general they bind preferentially to the (default) closed conformation, sometimes involving direct bonds to the MIDAS, but they do not induce conformational changes [4]. One counter-example is anthrax toxin, which utilizes a glutamate to engage the *bona fide* MIDAS motif of the "vWF A domain" of the collagen receptor, TEM8, in its open conformation [6]. There is also one clear example of gene transfer, in which the Gram-positive pathogen, *Streptococcus pneumoniae*, has an α-I domain inserted into the tip of its pilus, perhaps to act as a shear stress-activated adhesin for attachment to host cells [23, 36].

8.6 The β-I Domain and the Integrin Headpiece

The existence of a β-I domain was initially predicted based on the conserved and critical MIDAS-like sequence, DxSxS, and hydropathy plot comparisons with the α-I domain [34]; and later from more sophisticated sequence analysis [59]. The structure of the β3-I domain, contained within the αVβ3 crystal structure [64], confirmed that the basic fold and topology are very similar to the α-I domain, albeit with many large insertion/loops between β-strands, which had confounded conventional sequence alignment algorithms.

In contrast to α-I, the β-I domain is not folded independently, but packs rigidly against the α-subunit propeller, with the major ligand

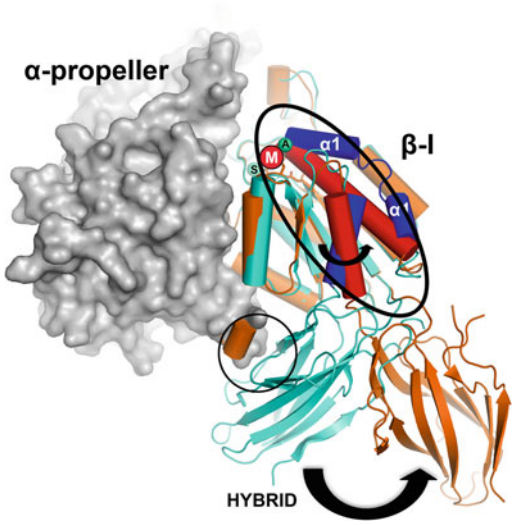

Fig. 8.4 Tertiary and quaternary changes triggered by ligand binding in integrins that lack an α-I domain. Ligand binding to the *β*-MIDAS motif (M) causes a shift of helix α1, which generates a rotation of α7 helix (*black arrow* within region *circled* in *black*) and a loosening of the contacts between the *β*-hybrid domain and the propeller. The *β*-hybrid domain is then free to swing by as much as 60° away from the α-propeller

recognition elements lying at the interface (see Fig. 8.4) [42, 64]. The *β*-MIDAS is similar to the α-MIDAS, except that the α-MIDAS threonine is replaced by glutamate. This difference likely explains the different cation specificities—in α-MIDAS, the smaller Mg^{2+} ion favors ligands lacking a formal charge; while in *β*-MIDAS, the larger Ca^{2+} ion favors multiple acidic ligands. The structure of α*V*β3 in complex with an RGD-style peptide shows that the Asp sidechain completes the coordination sphere of the MIDAS metal ion [65], as predicted. There are also further metal-binding sites adjacent to the *β*-MIDAS (the "ADMIDAS" and "SyMBS") that play important structural and possibly regulatory roles in ligand-binding and regulation [68].

Although Xiong et al. initially proposed the opposite, the conformation of the *β*-I domain in their unliganded crystal structure [64] corresponds to the closed conformation of the α-I domain. Soaking of RGD ligand into preformed crystals induced small changes within the *β*-I domain, but these were not propagated to the rest of the headpiece; i.e., they were frustrated by the constraints of the closed quaternary structure [37]. This situation is typical in crystallography: either the ligand binds and induces small changes constrained by the lattice, or it induces large changes that destroy the lattice.

However, Springer's group has recently discovered a rare exception to this rule, and report a crystal form of the αIIb*β*3 headpiece with large solvent channels in which the lattice tolerates (and/or adjusts to) a switch from the closed to the open conformation, involving an outward swing of the hybrid domain by ~40°. Preformed crystals were simply soaked with different concentrations/durations of an RGD ligand mimetic and different Ca^{2+}/Mg^{2+} ratios [69] (Fig. 8.4). This remarkable observations settles many questions with regard to head-opening, although the crystal structure of the headpiece in complex with a non-peptidic ligand is still lacking.

8.7 Quaternary Regulation in Integrins Lacking an α-I Domain

Takagi et al. [57] showed that the inactive (resting) form of the integrin α*V*β3, observed in physiological concentrations of Ca^{2+} and Mg^{2+}, is largely bent, and closely resembles the crystal structure, in which the C-termini of both chains are closely apposed. Based on the one case studied of an α-I domain integrin, the α*X*β2 ectodomain, it also adopts a similar (although distinct) bent default conformation [63]. Other integrins tested had a lower propensity to adopt the bent conformation; however, the experiments were performed with extracellular heterodimers truncated near the plasma membrane, so that they lacked the transmembrane helices and cytoplasmic tails that are known to contribute critically to the stability of the inactive conformation. By engineering a disulfide link between the α-subunit propeller and the EGF4 domain of the *β*-subunit (which are 4 Å apart in the bent (crystal) structure, but would be very far apart in the "standing-up" conformation), Takagi et al. further showed that integrin expressed on the cell surface was in a

low affinity state and could only be activated under reducing conditions.

The current model for integrins invokes a minimum of three distinct states: (i) bent, low affinity; (ii) standing-up, legs together, low affinity; and (iii) standing-up, legs apart, high affinity (see Fig. 8.1). The position of equilibrium depends on the concentrations and activation status of extracellular and intracellular ligands, as well as divalent cations. At the heart of the switch is an outward swing of the β-hybrid domain with respect to the β-I domain, by as much as 60° (Fig. 8.4). In αIIbβ3, the primary response to extracellular ligand binding is a concerted reorganization of the N-terminal helix (attached directly to the β-MIDAS) and the adjacent C-terminal helix. Rather than translate downward (as in the case of the α-I domain), the principle motion of α7 is a rotation about an axis close to the β-MIDAS, which is linked to the rotation of the β-hybrid domain. Thus, although some details may differ, the data support the prediction that the trigger for the integrin switch is similar in integrins that contain or lack and α-I domain: i.e., a subtle change in metal coordination at the MIDAS motif is linked to a reorganization of the I domain architecture that leads to quaternary changes toward an open, high-affinity state [33].

As noted above, these experiments were performed with truncated integrins and small peptide ligands. The nature of the trigger in the integrin head seems secure, but it remains to be seen how the quaternary changes are promulgated across the plasma membrane. Recent studies have shown that full-length integrin can be reconstituted into lipid nanodiscs and visualized by high resolution Electron Microscopy [12], so we should soon have an answer.

8.8 Quaternary Changes in Integrins Containing an α-I Domain

As noted above, in integrins that lack an α-I domain, the β-I domain and α-subunit propeller are the major recognition elements [42]. However, in integrins that contain an α-I domain, the β-I and α-propeller do not play direct roles in ligand recognition; instead they play important regulatory roles. This concept initially caused some confusion: thus, mutation of the β-MIDAS motif led to loss of iC3b binding to αMβ2 [3] which was initially interpreted as evidence for a direct role for the β-I domain in ligand; it now seems clear, however, that the mutation works allosterically, by preventing conformational changes in the α-I domain.

How does the quaternary organization of the integrin regulate the affinity of the α-I domain? We know that regulation occurs allosterically (rather than by steric masking of the binding site), since the α-I domain is a major antibody epitope. Hypotheses focused on the loss-of-function effect of mutating a Glu residue within a conserved ΦEGT motif (where Φ is any hydrophobic residue) at the end of the α-I domain C-terminal (α7) helix [1, 22, 66]; and it was suggested that the Glu could act as an intradimer ligand by completing the coordination sphere of the β-MIDAS motif. The first crystal structures of the αXβ2 headpiece (from Xie et al. [63]) were inconclusive: they showed the α-I domain in the closed conformation, but rather loosely attached to the rest of the headpiece. However, a recent structure of the αXβ2 ectodomain displays an activated α-I domain by virtue of a fortuitous crystal contact [47]. Although the rest of the $\alpha\beta$ headpiece is in the closed conformation, the predicted "internal ligand", Glu318, is observed coordinating the β-MIDAS motif with only minor compensatory movements in the β-I domain (Fig. 8.5). By contrast, the α-I domain adopts a fully "open" conformation, with the MIDAS threonine directly coordinating the metal and (what appears to be) a chloride ion completing the coordination sphere. The first half of the α-I domain α7 helix has shifted by $\sim$10 Å, as expected, but the remainder is unwound, thereby switching the orientation with respect to the headpiece. Thus, the crystalline environment seems to have created a hybrid molecule with a fully active α-I domain in the context of an inactive headpiece. It is possible that such a

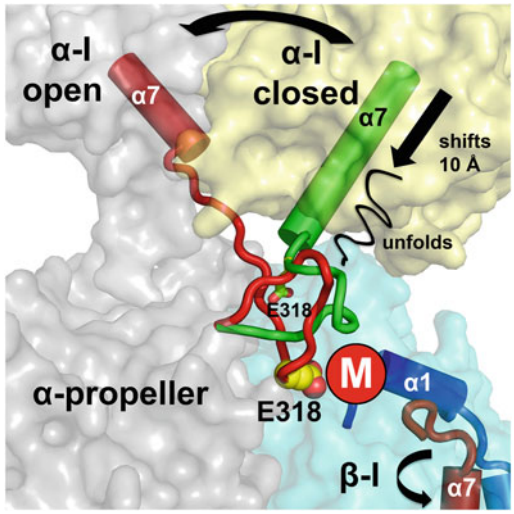

Fig. 8.5 Close-up comparison of the α-I domain-containing integrin head of αXβ2. In the open conformation, Glu318 acts as an internal ligand to the β-MIDAS that generates a 10 Å shift in the first half of helix α7 of the αI domain, while the second half of the helix unwinds, leading to a 30–40° rotation of the α-I domain about the propeller and β-I domain. The open α-I domain is stabilized by a crystal contact, and the the β-I domain remains principally in the closed conformation

hybrid state could exist in vivo, providing long-range, flexible and rapid (non-equilibrium) responses to the presence of ligand and/or mechanical stress; with a slow switch to the overall open (equilibrium) conformation occurring if the signal persisted.

8.9 Transmembrane (TM) and Tail Interactions

There are abundant biochemical and genetic data supporting the notion that interactions between integrin α- and β-TM helices and cytosolic tails help to hold the resting integrin in a low affinity conformation. In a classic study by Ginsberg and colleagues, a salt bridge between αIIb Arg995 and β3 Asp723 was shown to be necessary and sufficient to hold the integrin in its resting state [20]. A definitive structure of the αIIbβ3 tails in bicelles [32] reveals a remarkably stable conformation, in which the two helices pack closely together at the extracellular end; at the intracellular end they diverge, but the void is effectively filled by a highly conserved aromatic triplet that breaks the α-subunit helix and turns inward (Fig. 8.6). Isolated α and β subunit helices studies in bicelles show a remarkably well-conserved structure: the α-subunit is always orthogonal to the membrane while the β-subunit helix is always tilted. Recently, Ginsberg has shown that Lys 716β, whose Cα is buried in the membrane, can "snorkel" to the hydrophilic headgroups by extension of the Lys sidechain, and moreover that this residue is essential for maintaining the tilted helix and TM signaling [28, 55]. Recent studies on α-I domain integrins have yielded consistent results for isolated TM regions, and structures of αβ complexes are in progress [13]. The switch to the "open" conformation may entail a simple separation of the tails, which maintain their structural integrity and reassemble rapidly when the integrin returns to the low affinity state.

The penultimate question is how cytosolic proteins interact with the cytoplasmic tails of integrins, and the number of structural examples of protein domains bound to tail peptides (mostly β, but some α) has grown rapidly in recent years (see Table 8.1). It is clear that some proteins bind strongly enough to the β-tail to promote integrin activation. Talin was the first such molecule to be thus characterized, and remains the central player [8], although the number of additional contributors, such as kindlin and filamin [9], is growing fast. A model of talin activation is presented in Fig. 8.6. Talin sequesters the β-tail, breaking the critical R995α-D723β bond. It is also clear that phosphorylation of the β-tails provides a rapid means of switching between binding partners, and thus between cell migration and adhesion [14, 40, 44].

The final question is how the cytosolic activators generate force across the membrane. In the case of talin, recent work by Ginsberg's group suggests that dissociation of the tails, which have flexible linkages to the extracellular domains, is sufficient [29]. Key to this process is talin's ability to bind simultaneously to the integrin β-tail and membrane (Fig. 8.6), the latter providing a pivot point to force the two helices apart.

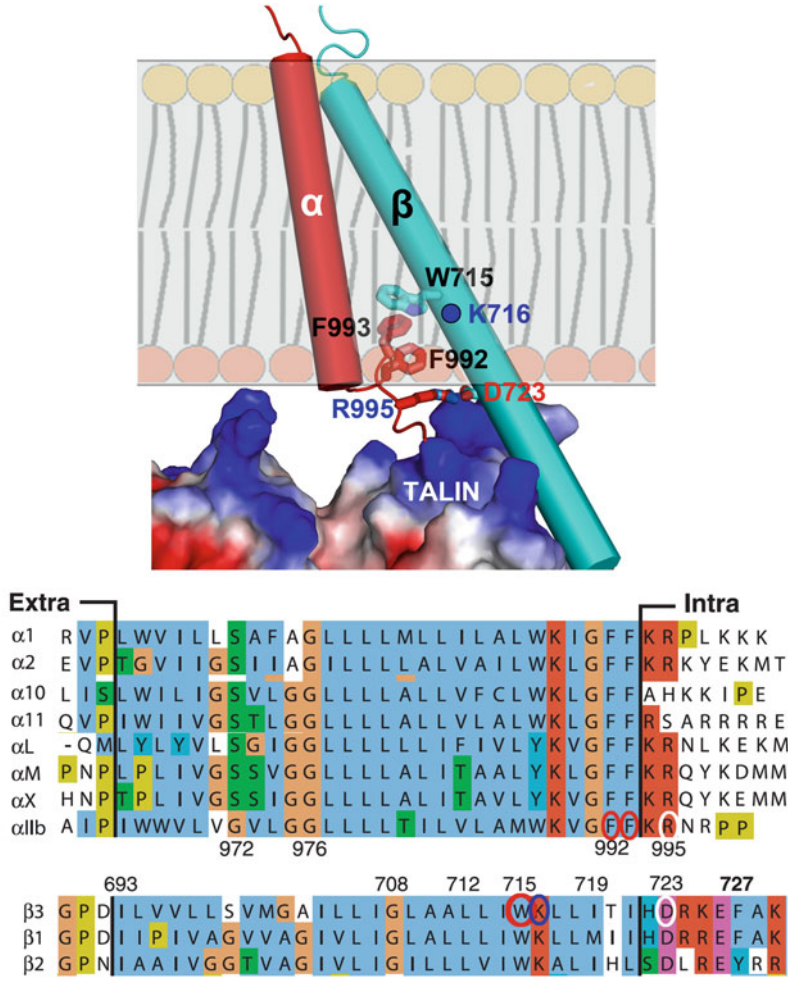

Fig. 8.6 Integrin-tail interactions. *Upper panel* Atomic interactions between the αIIb and β3 tails as revealed by NMR, melded to the complex of Talin2 and β1D tail. *Lower panel* Aligned sequences of α and β TM segments. Important residues in αIIbβ3 (and conserved in α-I domain integrins) are circled. See text for details

8.10 Perspectives

Structural and structure-function studies have revealed many of the major paradigms of integrin allostery underlying affinity regulation and bi-directional signal transduction. Notably lacking is the structure of an intact integrin bound to a physiological ligand in a membrane environment that would reveal the true "active" conformation of the molecule. EM studies show the greatest promise here, most likely using nanodisks. We are beginning to understand the structures of the TM helices and their cytosolic extensions, but the biophysics of inside-out signaling in particular requires further study. The role of mechanical force, whether of intracellular (actomyosin motors) or extracellular (shear flow in the vasculature) origin has not been discussed here, but its interplay with the chemical forces that attract cognate molecules is a fascinating field for current and future study [48]. Finally, this chapter has addressed the structural basis of affinity changes within

Table 8.1 Reported structures of integrins, 1996–present

Year	Protein	PDB code	Notes
Wild-type α-I domains			
1996	αM-I, Mg^{2+}	1IDO	
1996	αL-I, Mn^{2+}	1LFA	
1996	αL-I, Mg^{2+}	1ZOO, 1ZOP	
1996	αL-I metal-free	1ZON	
1997	αM-I, Mn^{2+}	1JLM	
1998	αM-I, soaks	1BHO, 1BHQ, 1IDN	Mg^{2+}, Mn^{2+}, free
1998	α2-I	1AOX	
2000	α1-I	1QC5	
2000	α1-I	1CK4	Rat
2000	αL-I	1DGQ	NMR structure
2003	αX-I	1N3Y	
2003	αL-I	1MQ9	High affinity form
I-like domains			
2004	A domain, Factor B	1Q0P	
2010	*Haemophilus* pilus	2WW8	
I domain-ligand complexes			
2000	α2-Collagen	1DZI	
2003	αL-ICAM1	1MQ8	
2005	αL-ICAM3	1T0P	
2008	αL-ICAM5	3BN3	
2013	αM-C3d	4M76	
2013	α1-Collagen	2M32	NMR/HADDOCK model
Engineered I domains/complexes			
2002	αM-I	1MIU, 1MQA	Ile switch
2003	αL-I	1MJN	Intermediate affinity
2003	αM-I	1MF7,1N9Z,1NA5	Modulatory mutants
2009	αL-I	3HI6	Disulfide-bonded intermediate
2011	α1-I	4A0Q	Activating mutation
2011	αL-ICAM-1	3TCX	Mutant high affinity I domain
2013	α2-Collagen	4BJ3	Mutant high affinity I domain
I domain-small molecule/FAB complexes			
2001	αL-LOVASTATIN	1CQP	
2004-2014	αL modulators	1RD4,1XDD,1XDG,1XUO,2ICA, 2O7N 3BQM, 3BQN,3E2M,4IXD,3F74,3F78	
2009/2010	αL-EFALIZUMAB	3EOA,3EOB,3M6F	

(continued)

Table 8.1 (continued)

Year	Protein	PDB code	Notes
2011	αM-FAB	3Q3G,3QA3	
Cytoplasmic Tail-protein complexes			
2003	β3-Talin (chimera)	1MIZ,1MK7,1MK9	
2005	β3-PIP-kinase	1Y19	
2005	αIIb-Filamin	2BP3	
2006	β7-Filamin	2BRQ	
2007	β3-TalinF3	2H7E	NMR
2007	β3-PIP-kinase	2H7D	NMR
2008	β2-Filamin	2JF1	
2008	β3-DOK1	2V76	
2008	β2-P-14-3-3	2V7D	P = Phosphorylation
2009	β1D-Talin2	3V9W	
2010	β3-shc-P	2L1C	
2012	αIIb-CIB1	2LM5	
2012	β1-Acap1	3T9K	
2012	β3-Src	4HXJ	
2013	β4-14-3-3	4HKC	
2013	β1-Acap1	4DX9	
Transmembrane Domains			
2008	β3	2RMZ,2RN0	
2008	αIIb	2K1A	
2009	αIIbβ3	2K9J	
2009	αIIbβ3	2KNC	
2011	β3	2KV9	S–S linked
2012	α2	2L8S	Detergent micelles
2014	αLβ2	2M3E	
2014	β3	2L91	
Cytoplasmic Domains			
2000	αIIb mutant	1DPQ	
2000	αIIb	1DPK	
2002	αIIbβ3	1M8O	
2002	αIIbβ3	1KUP,1KUZ	
2004	αIIbβ3	1S4W,1S4X	Micelles
2008	αL	2K8O	NMR
2011	P-β3	2LJF	Aqueous
2011	αMβ2	2LKE,2LKJ	
2011	P-β3	2LJD,LJE	
2012	αXβ2	2LUV	
α6β4 Intracellular domains/complexes			
1999	β4-FibIII pair	1QG3	
2008	β4-FibIII	2YRZ	NMR

(continued)

Table 8.1 (continued)

Year	Protein	PDB code	Notes
2009	β4-FibIII	3F7Q,3F7R	
2009	β4 Calx	3FQ4,3FSO,3H6A	
2009	β4-Plectin complex	3F7P	
Full-length integrins			
2014	αIIbβ3	4CAK	EM
2009	αVβ3	3IJE	Chimera?
Headpiece/ectodomain			
2001	αVβ3	1JV2	
2002	αVβ3-RGD	1L5G	
2002	αVβ3-Mn^{2+}	1M1X	
2004	αIIbβ3-inhibitor	1TYE	
2008	αIIbβ3 rerefined	2VC2,2VDK,2VDL,2VDM,2VDN,2VDO (supercede 1TY7,1TY3,1TXV,1TY5,1TY6)	
2008	αIIbβ3-Fibrinogen	2VDO,2VDP,2VDQ,2VDR	
2009	αIIbβ3	3FCS	"Complete ectodomain"
2009	αIIbβ3	3FCU	Open conformation
2010	αXβ2	3K6S,3K7L,3K72	
2011	αIIbβ3	3NID,3NIF,3NIG	Antagonist
2012	αVβ3	4DX9	Coiled-coil
2012	αVβ3	4G1M	Rerefined
2012	α5β1-RGD	3VI3,3VI4	
2012	α4β7	3V4P,3V4V	$\alpha\beta$ complex
2013	αIIbβ3-RGD	3ZDX,3ZDY,3ZDZ,3ZE0,3ZE1,3ZE2	
2013	α4β7	4IRZ	
2014	αXβ2	4NEH, 4NEN	

individual integrins. Lateral association (clustering) of integrins in the plasma membrane at sites of ECM contact also plays a major role in integrin signaling, and we still know little about its structural basis and regulation [5].

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Integrin Recognition Motifs in the Human Collagens

9

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Abstract

The best-known (fibrillar) collagens support cellular adhesion primarily through a subset of collagen-binding integrins, $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$, which have been shown to recognise a series of similar sequences. These contain Gxx′GEx″motifs (where x is a hydrophobic residue, x′ is usually O (hydroxyproline) and x″ is often R). Here, we review the variations within such sequences that support integrin reactivity, and their distribution across the 28 human collagens. The main basis for our understanding is the use of triple-helical, homotrimeric collagen peptides, but this work is far from exhaustive, and there is good evidence that heterotrimeric collagens where the sequence of interest occurs in two or even just a single chain may still support integrin binding. The fibrillar collagens I, II and III are rich in GxOGER motifs, whereas GxOGEK is more widely distributed, and less frequent in these three archetypal fibrillar collagens.

Keywords

Integrin · Collagen · Recognition motif · Peptide

9.1 Introduction

In this chapter, we review the interactions of the collagen-binding integrins, $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$, with the triple-helical domains of the collagen. Much detailed data has been accumulated from the use of the most abundant fibrillar collagens, types I, II and III, and from work in this and other laboratories where triple-helical synthetic peptides have been used to investigate the structure of complexes between collagens and integrins. The use of such (generally) homotrimeric peptides has established the rules that govern these interactions. An underlying assumption is that the integrin-binding motifs discovered in this way may be relevant to all homotrimeric, and some heterotrimeric triple-helical collagen molecules where the same sequence occurs. The general structure

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of the collagen family has been described clearly by Ricard-Blum [52], who also tabulates collagen-like proteins that contain triple-helical domains, but go by other names.

9.2 Collagen Structure

The collagens are defined by their repeated Gxx′ sequences, which can support assembly as a right-handed superhelix, the COL domain. Each of the three constituent polypeptide chains adopts a left-handed helical conformation, the polyproline II helix, with three residues per turn. Viewed axially, there is little space in the centre of the superhelix. This constraint dictates that glycine, lacking a bulky sidechain, should occupy every third position in the primary sequence, reviewed in [45]. Hence, both x and x′ sidechains are exposed on the superhelix surface where they may be free to interact with other molecules. The capacity to adopt the polyproline helical conformation arises from the distortion of the α chain backbone introduced by the cyclic iminoacids proline (P) and hydroxyproline (O) in the x and x′ positions respectively. Twenty eight human collagens exist, assembled from about 45 gene products, the family expressing surprising structural diversity [27]. The presence of a triple helix, as either a continuous COL domain or with non-helical interruptions, is their common defining feature. The collagens fall into several groups, with the fibrillar collagens, comprising types I, II, III, V, XI, XXIV and XXVII, being the prime example and the most abundant proteins within the vertebrate organism. Their fibrillar structure is achieved by the packing of the cord-like trimeric tropocollagen monomers in the typical quarter-staggered array, stabilised by electrostatic interactions between the sidechains of adjacent triple helices. The structure of the different collagen types, both as monomers and supramolecular assemblies, is nicely portrayed and reviewed by Ricard-Blum [52].

The fibrillar collagen genes encode precursors, procollagens, from which N- and C-terminal propeptides are trimmed by specific enzymes as the translated triple helix is secreted from the cell and assembles into the fibril. Processing leaves short unstructured telopeptide extensions at both the N- and C-terminal ends of the (generally single) COL domain. In contrast, the non-fibrillar collagens often contain several different classes of non-collagenous domains, notably VWF A, fibronectin III and thrombospondin domains, each with the capacity to support complex two- or three-dimensional network assembly. We have not attempted a detailed review of integrin binding to the non-helical domains of the non-fibrillar collagens, but there are many reports of such activity, such as within the C-terminal propeptide of collagen I which binds both $\alpha1\beta1$ and $\alpha2\beta1$ [14, 72], which have yet to be elucidated. Similarly endostatin, the C-terminal domain of collagen XVIII, interacts with $\alpha5\beta1$ by an unknown mechanism [51, 73].

Collagens as a group necessarily undergo post-translational modification, especially the hydroxylation of x′ proline and a proportion of x′ lysine residues. Subsequent crosslinking between triple helices occurs by condensation of hydroxylysine and lysine sidechains, adding to the stability of the fibril, whilst hydroxyproline supports hydrogen bonding through water molecules within the hydration shell surrounding the collagen monomer, sufficient to stabilise the initial assembly of both the helix itself and the fibril [49].

The collagen triple helices may be interrupted by stretches of non-helical sequence, separating the triple helix into multiple serial COL domains, conventionally numbered from the C-terminus. In the pro-form of the fibrillar collagens, such COL2 domains are found in collagens I, III and α2(V), whilst the remaining members of the sub-group also contain a smaller COL3 domain. In general, these are lost during procollagen cleavage, leaving just the COL1 domain and its telopeptide extensions, but this process is incomplete in collagen III, so that some unprocessed molecules occur on the surface of fibres where the COL2 domain may impede further fibre growth. Collagen IV and the fibril-associated collagens with interrupted

triple-helices (FACIT) are more extreme forms of such structures. Interruptions may take the form of extended insertions, which might be expected to contain secondary structure, or of small non-canonical insertions (interruptions or imperfections) into the COL domain of maybe three or four residues lacking glycine, or a deletion with just two intervening residues between conventional Gxx′ triplets.

The triple helix assembles with a one-residue offset between strands. As a consequence, three possible isoforms occur in heterotrimeric collagens, with, in principle, the single α2(I) chain of collagen I able to occupy the leading, middle or trailing position, and so that the trimer can present three different surfaces to protein binding partners such as integrins, as will be discussed further below. Firm knowledge of the order of strands in the heterotrimeric collagens is scarce. Whether the order is maintained through longer helical interruptions is not known, and in principle, needs to be defined case-by-case. From NMR experiments on a Gly/Ser substituted peptide [35], it seems likely that the order persists through short insertions or deletions with only slight perturbation resulting from the interruption. In the case of α1(VIII), for example, the COL1 domain contains eight occurrences of a single-residue deletion (resulting in a "two-residue triplet"), and the corresponding α2(VIII) contains eight equivalent compensating deletions usually four residues towards the C-terminus, that will allow the maintenance of an essentially linear COL domain with only minor deviation. In contrast, the FACIT collagen IX has unequal longer insertions in its α-chains that will introduce a flexible kink between the COL2 and COL3 domains.

9.3 Integrin I-Domain Structure

The vertebrate integrins are a family of 24 heterodimeric trans-membrane cellular adhesion molecules, with carefully regulated expression and affinity, reviewed in [4]. Integrins mediate either cell–cell interactions, binding counter-receptors such as ICAM and VCAM, or cell–matrix interactions [24]. The two matrix molecules best-known in the latter context are fibronectin and collagen. Fibronectin is a ligand for both β1 and β3 integrins, with α5β1 and αvβ3 as its most widespread receptors, whereas collagen is considered to interact directly only with that subset of four β1 integrins, α1β1, α2β1, α10β1 and α11β1, which is distinguished by the presence within the α-subunit of an inserted, or I, domain [4]. These integrins are discussed in detail in Chaps. 2, 3, 4 and 5 of this volume. The α-I domain adopts the dinucleotide-binding, or Rossman, fold [17], and its evolution is discussed in Chap. 1. This structure, the prototype for which is the von Willebrand factor A domain, is found in intracellular signalling species such as G protein α-subunits and in extracellular adhesive proteins including the terminal extensions of the non-fibrillar collagens [13]. Under physiological conditions, the integrin α-I domains, though not necessarily all other A-domains [6], constitutively co-ordinate a divalent cation, Mg^{2+}, in their metal ion dependent adhesion site (MIDAS) which is the focus of their interaction with collagens [18]. Of the four collagen-binding integrins, α1β1 and α2β1 have been studied for almost three decades whilst the properties of both α10β1, which was purified more recently using its capacity to bind collagen II [5, 7, 8] and α11β1 (reviewed in Chap. 5) are still not fully investigated. Although α1β1 and α2β1 share most sequence identity, the binding properties of α1β1 and α10β1 appear most similar, but distinct from those of α2β1 and α11β1, which also appear to share some functional redundancy [4]. Integrin α1β1 is considered a selective ligand for collagen IV, and α2β1 for collagen I [67, 68]. This indicates differential ability to interact with specific collagen types, and by inference therefore, with different motifs within the collagens. Crucially, the tissue distribution and temporal expression of the integrins may differ significantly, with α10β1 being most abundant in cartilage, for example. Transcripts of all four are increasingly being detected in diverse settings, where functional significance is as yet unknown. In this volume, Chap. 10, Heino summarises the

integrin-reactivity of the different collagen types.

The possibility that leukocyte integrins (also with I domain-containing α subunits) can bind collagen arises from time to time. Collagen is featured in "Integrins at a glance" as a ligand for αxβ2 [24], and recently Lahti et al. showed binding of leukocytes and recombinant I domains to collagens and to a GFOGER-containing collagenous peptide [33]. In our hands, these I domains do indeed bind to such peptides, but weakly, and non-specifically: where cation dependence occurs in our data, similar binding occurs to the control peptide, GPP-10, which lacks any integrin motif (Fig. 9.1). To our minds, therefore, this may represent a different mode of binding than that regarded as the canonical mechanism [18].

9.4 Regions of Collagen That Bind Integrins

In this chapter, we will focus upon the binding of motifs within the COL domains to integrins, where triple-helical structure is a pre-requisite for binding activity.

Michael Barnes [20, 21, 30, 37, 39, 66] and Sam Santoro [15, 55–57, 62] were the most prominent workers in the field during the last 15 years of the twentieth century. Both laid the foundations for the present understanding, and used the human platelet collagen receptors as an established and accessible cellular model for collagen receptors in general, together with a human fibrosarcoma cell line, HT1080, that is still considered to express integrin α2β1 as its major functional collagen-binding integrin. The three other family members may occur at low copy number in both cell types, but the cellular adhesion to collagen is virtually abolished by α2 subunit-specific blocking antibodies.

Separately, Barnes [38] and Santoro [63] each mapped α2β1 onto the cyanogen bromide peptide, α1(I) CB3, identifying α2β1 as a key receptor that supported strong adhesion of platelets to collagen, but which was not sufficient to activate the platelet. Santoro found integrin-binding activity to be restricted to CB3 [63], whilst Barnes was able to show sites within several other CB peptides, although CB3 contained the highest affinity ligand for α2β1. Santoro used chemical derivatisation to show that specific reactivities within the intact collagens were responsible for binding to α2β1 and to an activatory platelet receptor, and subsequently to propose a linear tetrapeptide, DGEA, as an α2β1 recognition motif [61]. In turn, Barnes extended his reductive use of CB peptides, which yielded low-resolution maps of integrin binding sites in collagens I and III, to the development of a small library of overlapping, triple-helical synthetic peptides which encompassed the whole of α1(I)CB3 [30, 31]. By this method Barnes identified the sequence GFOGER as what remains the highest-affinity triple-helical ligand for α2β1 discovered to date.

These two contrasting reactivities remain in the literature: DGEA, reported to be active in linear form [61], and GFOGER, active as a triple-helix but not as a shorter, linear peptide [31]. In our hands, neither linear nor triple-helical DGEA bound the resting platelet, a point to be discussed further below. Nonetheless, using either DGEA or GFOGER as a title keyword to search PubMed returns ~50 papers for each attesting to their integrin-reactivity, but to date no complex of an integrin I domain with DGEA has been deposited in the Protein Data Bank, although three complexes between integrin I domains and GFOGER or a similar ligand are present (1DZI, 4BJ3 and 2M32).

9.5 Collagen Sequences That Bind Integrins

9.5.1 GxOGER and Related Ligands in the Fibrillar Collagens

Both Barnes and Emsley promptly recognised GFOGER as just one of a series of integrin-binding motifs occurring in the fibrillar collagens [18, 31], as did Hook's group, who reported

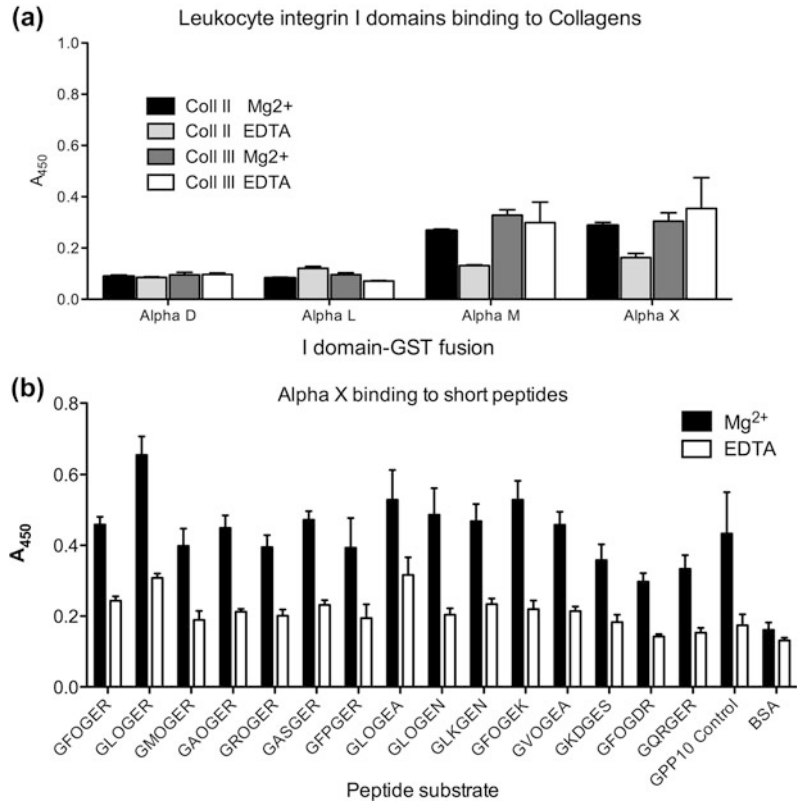

Fig. 9.1 **a** The ability of the recombinant I domains of leukocyte integrins to bind to collagen II and III monomers in ELISA-like assays is shown. With the exception of αM and αX applied to collagen II, there was no diminution of binding in the presence of EDTA. **b** Explores the capacity of triple-helical peptide motifs, in GPC–$[GPP]_5$–Gxx′GEx″–$[GPP]_5$–GPC format, to support leukocyte integrin I domain binding. Note that these values are low, and the Control peptide, GPP10, displays almost as much activity as the authentic motifs, and is also EDTA-sensitive. Compare amplitudes with the data in Fig. 9.2, where α2 I domain is used under the same conditions

a weakly-binding GASGER in collagen I and subsequently GROGER in collagen III [29, 74]. These motifs generally have a hydrophobic residue at position x in the GxOGER generic sequence. Siljander summarised their occurrence at specific loci within the D-periods of a wide range of fibrillar collagens, (D1: GLOGER; D2: GAOGER; D3: GFOGER in collagens I and II, GAOGER in III; GMOGER in collagens I, II and III) [59]. Beyond D3, conservation is less good, with weakly-binding motifs such as GAOGER present in some collagens and GQRGER or GLSGER in others. The activity of several of these sequences has been tested using synthetic peptides, and subsequently, Raynal extended this approach by synthesising the 27-residue peptide library, Collagen Toolkit III, and was able to confirm the activity of GROGER and to discover GLOGEN as an integrin-binding motif, later shown to be a preferred ligand for $\alpha 1\beta 1$ [50].

GROGER, quite a good ligand for $\alpha 2\beta 1$, unexpectedly contains the positively-charged arginine in place of the bulky hydrophobic residue that was thought to be essential. It seems quite likely that the three-carbon stem of its sidechain might fulfil the same function, supporting hydrophobic contact with the I domain surface, but this requires confirmation through structural study.

GLOGER was identified as a good ligand for $\alpha 1\beta 1$, as good as GFOGER, whilst GLOGEA

seems similarly effective. It is therefore not surprising that GLOGEN proved to be a higher-affinity selective ligand for $\alpha1\beta1$, recently identified in this context, along with GVOGEA, a weaker but specific $\alpha1\beta1$ ligand [23]. Subsequently, a low-resolution SAXS structure of a complex between $\alpha1$ I domain and a GLOGEN-containing peptide has been reported, with the interesting demonstration of a 2-to-1 I domain-to-peptide complex [12]. To achieve this outcome, an activated form of $\alpha1$ I, E317A, was used, similar to the equivalent active form of $\alpha2$ I domain, E318W [2], also shown previously to form a 2-to-1 $\alpha2$ I-to-GFOGER crystal complex [9]. Both I domains supported 2-to-1 complexes in solution, suggesting that this is not merely a crystallisation artefact. Whether such complexes can occur in nature is debatable, but might represent a means of cell–cell adhesion, using a single strand of collagen as an intermediate bridging ligand. Such single triple helices might be found in non-fibrillar collagens such as collagen IV, or after dispersion of collagen fibres during tissue resorption or remodeling during wound repair. Whatever the significance of these 2-to-1 structures, they reveal a lower affinity mode of binding that may become operative when an integrin is activated, discussed further below.

Inspection of sequence, based on knowledge accrued since the first reports in 2000, has proved valuable in identifying potential integrin-binding sites in the collagen family at large, sometimes supported by synthesis of corresponding triple-helical peptides. The fibrillar collagens, I, II and III have attracted most attention, not least because the development of the peptide Toolkits renders their study straightforward. No such reagents exist at present for other collagens. The sequences of the fibrillar collagens, V, XI, XXIV and XXVII, are much less rich in GXOGER motifs than the more familiar I, II and III (see Table 9.1), reflecting their different evolutionary paths after the emergence of the ancestral collagen I, and the restricted expression of collagen XXVII to embryogenesis [47].

9.5.2 GxOGEK

Conservative substitution of K for R within triple-helical integrin ligand peptides revealed a similar motif, GFOGEK, as a useful ligand for $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ [23, 75], suggesting that it might prove to be representative of a series of GxOGEK homologues. Such sequences, like GxOGER, are widespread within the collagens as a whole.

GxOGEK motifs are rare in collagens I, II and III, with single occurrences in $\alpha2$(I) and at a discrete conserved locus in $\alpha1$(II) and $\alpha1$(III), seven triplets before the collagenase cleavage site. Collagen V lacks established GxOGER motifs, except for GMOGER in $\alpha2$(V), at loci conserved with integrin sites in D1 and D3 of collagens I, II and III. At the nearby locus in D3 where GFOGER occurs in collagens I and II, collagen V contains GNOGER, that deserves investigation. Collagen V also contains several GLOGEx″ motifs that might offer a site to $\alpha1\beta1$, including GLOGEK at a locus conserved in its $\alpha1$ and $\alpha3$ chains. Few other of the OGEK motifs that are relatively abundant appear to be promising integrin sites, lacking hydrophobic side-chains at the x position. GLOGEK in $\alpha2$(V) and GIOGEK in $\alpha3$(V) are the exceptions. Collagen V has a role in pericellular collagen fibrillogenesis in various tissues [60, 64, 71], and can support $\alpha11\beta1$-mediated cell migration [48].

Collagen XI, a co-constituent of cartilage fibres along with collagen II, is more promising, with a GFOGER site aligned in its $\alpha1$ and $\alpha2$ chains at a central D3 locus conserved with that in collagens I and II. GLOGEA in $\alpha1$(XI), that may bind $\alpha1\beta1$, aligns with GLOGES in $\alpha2$(XI), located in D1 near the GLOGEN and GLOGER sites in collagens I, II and III. This will most likely reconstitute an integrin site similar to the GFOGER/GPOGES site in $\alpha1$(I) and $\alpha2$(I). In Chap. 4, Lundgren–Åkerland describes binding of $\alpha10\beta1$-expressing cells to collagens II and XI, as well as to GFOGER and GLOGER-containing peptides.

The remaining fibrillar collagens, XXIV and XXVII, lack established integrin-binding motifs

Table 9.1 Integrin recognition motifs in all human collagens

	Chain	GER	GEK	GLOGE	RGD	Known and prospective integrin motifs (listed from N-terminus)
Fibrillar	Iα1	11	1	1	2	GROGER, GLOGER, [a]GFOGER, GMOGER, GQRGER, GASGER
	Iα2	8	3	2	2	GROGER, GLOGER, GLOGER
	II	11	4	1	3	GLOGER, GVOGEA, [a]GFOGER, GMOGER, GQRGER
	III	14	4	1	1	GROGER, GLOGEN, [a]GAOGER, GMOGER, GLSGER
	Vα1	7	14	3	2	*GLOGEK*, *GLOGEO*, *GLOGEG*
	Vα2	10	3	1	7	GMOGER, [a]*GNOGER*, GMOGER, GQRGER
	Vα3	7	14	0	3	
	XIα1	6	14	1	0	GLOGEA, [a]GFOGER
	XIα2	8	14	2	3	[a]GFOGER
	XXIV	5	7	4	0	*GNOGER*, [a]*GLOGEO*
	XXVII	3	4	2	3	[a]*GLOGEO*, GLOGEA
Network	IVα1	6	21	3	3	GFOGER, **GDQ**, *GLOGEK*, *GLOGEK*, *GLOGEK*
	IVα2	6	6	3	9	*GLOGEM*, **GRA**, *GLOGEV*, GAOGER, *GLOGEK*
	IVα3	4	9	1	6	*GLOGES*, GFOGER
	IVα4	5	11	1	8	GLOGEA, GFOGER, GFOGER
	IVα5	6	14	2	0	*GLOGEK*, GFOGER, *GLOGEO*
	IVα6	3	10	1	3	*GLOGEK*, *GLOGEL*
	VIIIα1	0	1	0	0	
	VIIIα2	1	0	0	2	*GVOGER*
	X	5	1	0	0	*GKOGER*, GFOGEK, GROGER
FACIT	IXα1	3	3	1	0	*GLOGEL*
	IXα2	2	4	2	0	*GLOGEI*, *GLOGEK*
	IXα3	4	3	0	2	GMOGER
	XII	1	1	0	2	*GLOGEK*
	XIV	3	2	0	1	
	XXVI	7	17	0	1	
	XIX	6	7	1	1	*GLOGEH*, *GIOGEK*
	XX	2	2	0	0	
	XXI	1	4	0	0	
	XXII	8	14	4	2	*GLOGEV*, *GLOGEI*, *GNOGER*, GLOGEN
TM	XIII	4	10	0	0	
	XVII	1	3	0	0	
	XXIII	0	1	0	2	
	XXV	3	10	0	0	
Multiplexins	XV	1	9	0	0	
	XVIII	3	4	1	1	*GLOGEO*

(continued)

Table 9.1 (continued)

	Chain	GER	GEK	GLOGE	RGD	Known and prospective integrin motifs (listed from N-terminus)
Other	VIα1	4	6	1	3	*GLOGEK*, GAOGER
	VIα2	1	4	0	5	
	VIα3	4	3	0	5	GFOGEK, GAOGER
	VIα5	0	3	0	1	
	VIα6	2	3	1	1	*GLOGEM*
	VII	22	17	2	3	GAOGER, GLOGER, GFOGER, GROGER, GLOGER, GAOGER
	XXVI	1	2	1	0	*GLOGEM*
	XXVIII	6	2	1	1	

[a] Indicates conserved high-affinity locus in D-period 3 of the fibrillar collagens. Alignment of other sites is approximate. **Bold** indicates the composite site in α1(IV) and α2(IV). *Italics* indicates prospective sites, not tested to date. In the fibrillar collagens, some known low-affinity sites (GAOGER) are omitted to conserve space

such as GxOGER, except the untested GNOGER in α1(XXIV). Plausible GLOGEx″ α1β1-selective motifs are also found in α1(XXIV), with Q, V, O and D as x″. Similarly, collagen XXVII contains single occurrences of GLOGEO and GLOGEA, prospective α1β1 ligands.

9.6 Sites in the Non-fibrillar Collagens

9.6.1 Network-Forming Collagens, IV, VIII and X

The non-fibrillar collagens are much less rich in defined GxOGER integrin-binding sites, and where such sites have been proposed, their exact location within the triple-helical domains is more often by inference than experiment. Parkin et al. have prepared a collagen IV interactome [44], and mapped putative integrin sites within the three different heterotrimers of collagen IV (α1α1α2, α3α4α5 and α5α5α6), using the location of GxOGEx″ motifs as a guide. A point of interest is that few motifs are aligned in all three constituent chains. One exception, in a region described as an endothelial cell binding domain in the most abundant form of basal lamina collagen IV, α1α1α2, contains a GFOGER/GFOGER/GLOGEM locus near to the disperse α1β1 site identified by Kuhn's group, discussed further below. Other potential sites, GLOGEx″ being a prime example that is represented in all six α chains, often occur unsupported in the other chains of the heterotrimer, but nonetheless form credible sites for both α1β1 and α2β1; critical interactions are thought to involve just one GxOGEx″ glutamate within a triple helix, with crucial ancillary stabilisation from hydrophobic x or positively-charged x″ residues. In α3α4α5, both GLOGEx″ and GFOGER occur unsupported in different loci in each α chain, but the adjacent chains contain x hydrophobic residues that may contribute to binding. OGEK triplets are sparse, with GLOGEK and GIOGEK in α5(IV) the most promising, although GEK occurs many times. These, along with GFOGER, form promising loci in the α5α5α6 form of collagen IV, but the α6 chain contributes just one each of GLOGEK, GFOGEK and GLOGEx″ as putative integrin sites.

Kuhn's group reported and have researched extensively a disperse site for α1β1, comprising GPOGDQ triplets in α1(IV) and the aligned GAKGRA triplet in α2(IV). The nature of this site remains enigmatic: one of the α1 chain aspartate residues co-ordinates the metal ion in the α1 I domain MIDAS, yet a competent recombinant α1 I domain is unable to bind a model homotrimeric peptide containing the sequence GFOGDR [23]. The critical but ancillary role of the hydrophobic F residue, important in defining the affinity of peptides for

α2β1, seems insufficient to support the GDR triplet in binding α1β1, despite GFOGER being a moderately good ligand for α1β1. In the native α2(IV) collagen chain, the lysine of the GAK triplet preceding GRA has been proposed to have a critical role, by forming a salt bridge with the I domain surface [53].

Chin models a slightly different peptide-I domain relationship [12], using low resolution SAXS- and NMR-derived structures to direct the docking programme HADDOCK, and suggest that the peptide, triple-helical GLOGEN, sits more centrally within the binding trench in α1 than in α2, where the corresponding GFOGER is described as binding to the edge of the equivalent trench in the α2 I domain. Part of the rationale for this may be a steric clash of the bulkier sidechain of the x″ arginine in GER-containing motifs, explaining why GEN may be a preferred ligand for α1β1. In line with these ideas, Seo modelled and expressed an unnatural sequence, GFPGEN, that they found to be selective for α1β1 [58]. Emsley described the α1 I domain as having a flatter, more open MIDAS, more readily able to receive a short co-ordinating aspartate than α2 I domain [18]. Further work to extend these concepts to include α10β1 is called for.

Both the α1 and α2 chains of collagen VIII are devoid of defined integrin motifs, although GVOGER in its α2 chain, by analogy with GVOGEA in collagen II, presents a possible binding motif. Turner showed that endothelial cell attachment to collagen α2(VIII) homotrimers was partially mediated by α2β1, using anti-α2 blocking antibodies [69]. Anti-β1 completely inhibited adhesion, implying the presence of other collagen-binding integrins. Of general note, a cyclic RGD peptide had no effect on cell adhesion to this collagen preparation despite the presence of two RGD motifs within the COL domain. Here, as in other collagens, triple-helical RGD was not recognised by the relevant integrins although the latter were present and competent to bind fibronectin. Adiguzel note the upregulation of collagen VIII in atherosclerotic tissue [1], where it may support vascular smooth muscle cell migration, and identify β1 integrin-mediated signalling to the small GTPase, RhoA.

Collagen X contains one known and one possible GxOGER motif, GRO and GKOGER, along with the proven GFOGEK.

9.6.2 FACIT Collagens, IX, XII, XIV, XVI, XIX, XX, XXI and XXII

The heterotrimeric collagen IX, a FACIT collagen associated with collagen II fibres, was reported to express strong integrin-binding activity, tested on all four collagen binding I domains, compared to other collagens [28] and showing similar high affinity for α1, α2 and α11, but rather weaker for α10. (See also Chap. 4 in this volume.) This might be attributed to its aligned GLOGEL and GLOGEI motifs which occur in the (N-terminal) COL3 domain of the α1(IX) and α2(IX) chains. Collagen IX is not rich in established motifs; the COL2 domain contains GLOGEK in α2(IX) and GMOGER in α3(IX). It may be that these GLOGEx″ motifs are selective for α1β1. Whilst the patency of GFOGER in collagen I (and by inference, in collagen II) has been questioned by Orgel's group, GFOGER being proposed to be systematically buried within the complex twisted structure of the fibre [46], the presence of strongly integrin-binding FACIT collagens may compensate for this effect, if it does indeed occur.

Collagen XII presents only the untested GLOGEK. Although it is proposed that integrin-mediated tension may stimulate collagen XII expression, its capacity to bind integrins directly is unknown [65]. It is worth noting that the non-collagenous domain 3 (NC3) of collagen XII contains several VWF A domain structures with intact DxSxS MIDAS motifs. This raises the interesting possibility that the affinity of the FACIT collagen XII for the surface of a collagen fibre may include a cation–dependent interaction between the its VWF A domains and integrin-binding motifs in the fibrillar collagen. A similar VWF A domain-mediated mechanism of

collagen–collagen interaction (dimerisation) has been described for collagen VI [3] in this case utilising the COL domain sequence GSOGER as a counter-ligand. The ability of collagen XII to regulate the biomechanical properties of associated fibres has been attributed to its NC3 domain [40].

Collagen XIV contains no defined integrin motif, although GMOGEK and GTOGER represent untested possibilities. Collagen XVI, although rich in GEK and GER triplets, lacks any OGEK or OGER motifs, but Eble et al. [16], propose GLQGER and GIKGER, but not GGKGER and GKAGER, as integrin-binding in collagen XVI. Two of its interrupting sequences contain RGD motifs, raising the possibility of $\alpha5\beta1$- or $\alpha v\beta3$-mediated adhesion to these less-structured NC domains. However, Eble concluded that these motifs, along with one RGD triplet that lies firmly within a COL domain, do not contribute to cell-binding activity, reinforcing the conclusion that RGD is cryptic in collagens when located in their COL domains.

Collagen XIX presents two possible motifs, GLOGEH and GIOGEK, again untested.

Collagens XX and XXI, both designated FACITs, contain four or five of either GER or GEK motifs, none preceded by O, and therefore lack obvious integrin-reactivity. In contrast, collagen XXII, with a much longer interrupted triple-helix, contain 14 GEK motifs, possible integrin-binders, including GROGEK, and eight GERs, including GEOGER and GNOGER.. Most telling, four GLOGEx″ motifs occur, including GLOGEN, now shown to be an $\alpha1\beta1$-selective motif [23]. Koch applied HACAT keratinocytes ($\alpha2\beta1$-expressing) and WI-26 fibroblasts (which express both $\alpha1\beta1$ and $\alpha2\beta1$) to collagen XXII, the latter binding more efficiently [32]. It is tempting to conclude that the sequence, GLOGEN was central to this outcome.

9.6.3 "Other" Collagens

Amongst the collagens VI, VII, XXVI and XXVIII (undesignated by Ricard-Blum), only collagen VII is as rich as the archetypal fibrillar collagens in GxOGER motifs, where x is A and L (twice each), F or R. This suggests a marked propensity to bind integrins. Saelman et al. report $\alpha2\beta1$-dependent adhesion of platelets to collagens including VI and VII [54]. However, Chen et al. reported $\alpha2\beta1$-mediated attachment to the NC1 domain of VII that was RGD-independent and survived denaturation [11], and Liebert et al. [36] observe co-localisation of collagen VII with $\alpha6\beta4$, not known as a collagen receptor. This interaction may regulate laminin-332 organisation during wound healing [43]. Whether the collagen VII COL domain, although an attractive candidate, expresses the anticipated strong integrin reactivity remains to be established.

Of the others, the short ($\sim$100 triplet) $\alpha1$(VI) and $\alpha3$(VI) COL domains each contain only GAOGER and either GLOGEK or GFOGEK, whilst XXVI contains a single GLOGEM motif and XXVIII contains a single GVOGER motif, mentioned elsewhere above.

9.6.4 Multiplexins, Collagens XV and XVIII

Collagen XV lacks obvious motifs, although contains several GEK triplets, whilst collagen XVIII contains a single GLOGEO motif. Consistent with this, collagen XV has been found not to support cell adhesion [26], but instead to bind other matrix proteins (FN, LN and VN) that will offer RGD-dependent cell-binding activity. Halfter et al. report little cell binding activity of collagen XVIII [22].

9.6.5 Transmembrane Collagens, XIII, XVII, XXIII and XXV

The transmembrane collagens are intriguing, since they offer novel and barely-explored opportunities for cell–cell interaction. Collagen XIII lacks obvious integrin sites, with four GER and several GEK triplets, but none preceded by

O. Nonetheless, $\alpha1\beta1$, though not $\alpha2\beta1$, binding is reported [42]. Integrin reactivity has also been identified for collagen XVII [41], which when transfected into suitable cell lines, also binds the non-integrin immune collagen receptor, LAIR1 [34, 42]. Again, obvious integrin-binding motifs are absent.

Collagen XXIII contains the low-affinity motif, GASGER, but no other obvious integrin-binding activity amongst the sundry GEK triplets, suggesting weak or absent integrin reactivity. Veit et al., using bacterially expressed foldon-peptide collagenous materials, suggest that the integrin reactivity they observed in collagen XXIII does not reside in GTSGER or GEKGER [70].

Collagen XXV, similar in most respects, lacks even low affinity GER motifs.

9.7 Recognition of Integrins by Multiple Collagen α-Chains

The first collagen peptide-I domain complex [18] showed that two strands of a homotrimeric GFOGER peptide, the leading and the middle strand, were involved in integrin binding. The middle strand was crucial, containing the glutamate responsible for much of the binding energy. As described, however, the contribution of the hydrophobic phenylalanine was also critical, and the identity of x in GxOGER-containing peptides defines an affinity series at the level of binding assays [23, 50, 59], and in determining the ability of a cell to migrate across a peptide coated surface [19]. The positively charged arginine residue also forms an important salt bridge with corresponding negative charges on the I domain surface, in α2 I domain at least. The leading strand, however, makes several significant contributions, including further hydrophobic bonding and a less intimate charge–charge interaction with its F and R residues respectively. These interactions have been detailed by Emsley and others and need little reiteration here. From this body of work it seems unlikely that a heterotrimer containing a GxOGER motif in just a single strand would support high-affinity interaction. This remains to be explored experimentally.

9.8 Effect of Cellular Activation and Integrin Binding

The recent publication of a new 2-to-1 I domain-to-peptide structure [9, 12] shows that a second binding mode is possible. In this receptor–ligand complex of α2 I with GFOGER, one copy of an activated form of α2 I (E318W) adopts a relationship to two strands of the peptide that is essentially identical to that described by Emsley, although in this iteration, the trailing strand takes the primary role by housing the crucial glutamate. Other equivalent supporting interactions are provided by the middle strand. The second copy of the I domain, however, binds mainly to just one strand, the leading strand, with its glutamate conventionally co-ordinating the MIDAS. There is no arginine available in an adjacent strand to meet the requirement for salt-bridging, and, by the same token, no phenylalanine to offer additional hydrophobic bond support. Proline in the peptide flanking sequence fulfils this function. The 2-to-1 complex was sufficiently robust to survive gel filtration, but not when a lower-affinity peptide, GMOGER, was used. Crucially, an active mutant of α2 I, E318W, was used to prepare the complex; wild type would not support stable complex formation in solution with either peptide. A similar complex was published subsequently between the preferred ligand for $\alpha1\beta1$, GLOGEN, and the corresponding E317A form of α1 domain, where, presumably, similar considerations apply [12].

This work suggests that, upon activation, interactions of integrins with lower affinity sequences can become useful. For $\alpha2\beta1$, the affinity series, $x = F > L \geq R > M > A$ in GxOGER-containing motifs appears to hold well in different cell adhesion studies, e.g. using platelets and HT1080 cells [19, 59]. With the latter, although resting cell adhesion was virtually negligible in relatively stringent static

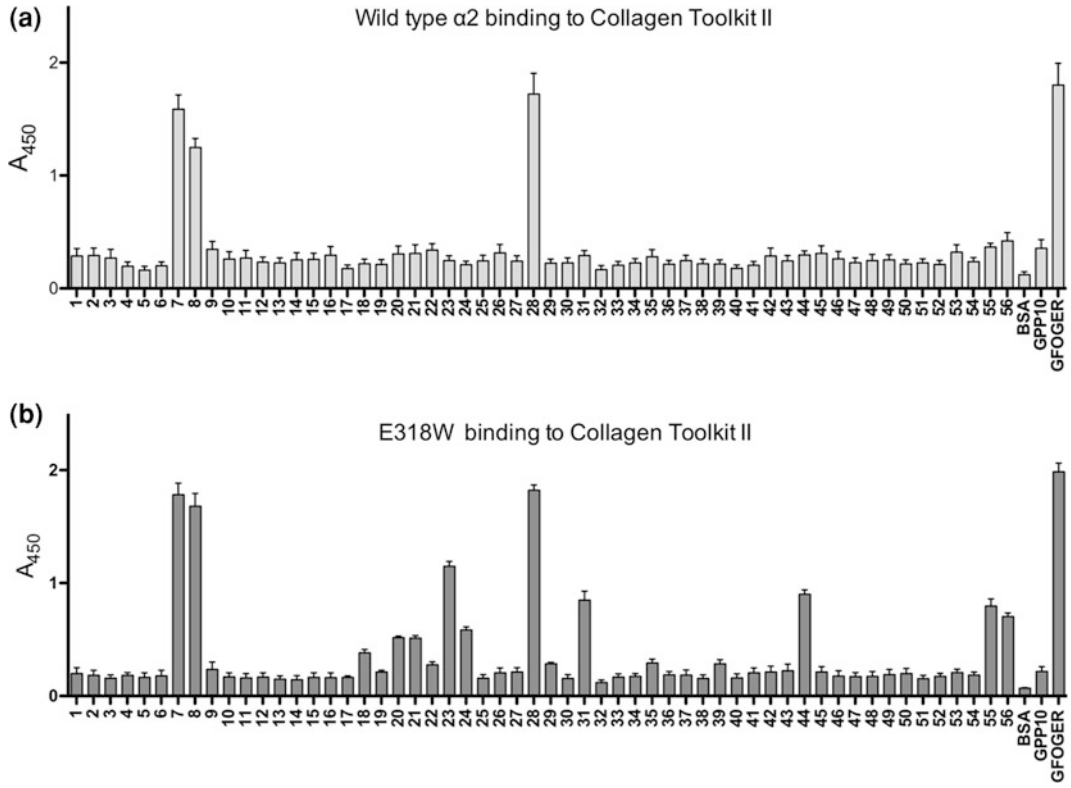

Fig. 9.2 **a** The ability of wild type α2 I domains to bind to Toolkit II is shown. Wild-type binds well to just three peptides, II-7, II-8 and II-28. GLOGER occurs in the overlap between II-7 and II-8; GFOGER in II-28. **b** Shows the equivalent experiment using the more active I domain, E318W, which, in addition, binds well to established motifs, GMOGER in II-31, GQRGER in II-44, and less well to GAOGER in II-18. Other binding activity remains undefined, but it may be significant that II-23 and II-24 share GKAGEK in their overlapping sequence. All other weakly-binding peptides include GEx″ motifs. (Reproduced from Carafoli et al. [9] under the creative commons license. See http://creativecommons.org/licenses/by/3.0/)

adhesion assays (with multiple wash steps) the capacity of cells to migrate upon GAOGER surfaces was much greater than on GFOGER. Thus, a tight-binding surface may not be what is needed if cells are to invade, repopulate and repair a wound quickly and successfully.

The use of activated I domains provides other useful insights; lower-affinity peptides, with corresponding lower-affinity motifs, appear positive on Toolkit maps, (see Fig. 9.2) but binding curves (Fig. 9.3) indicate that the affinity of such motifs may be one or two orders of magnitude lower than the best motif, GFOGER [9]. Nonetheless, these weak interactions may become more important in the context of an activated cell. It is important to note that whilst the selectivity of the activated form of the I domain is decreased (more Toolkit peptides become positive), binding affinity for the established peptides increases. This indicates that the activated I domain surface is able to make a greater number of contacts with the peptides than the resting integrin, suggesting greater plasticity of its binding surface. (As an aside, DGEA, which occurs in Toolkit peptide II-03, does not bind either wild type or E318W I domain.)

Taken together, these data support the idea that weaker motifs, or motifs present in only a single strand of a heterotrimeric collagen, may be valuable in the context of cellular behaviour that requires weaker interactions, and where

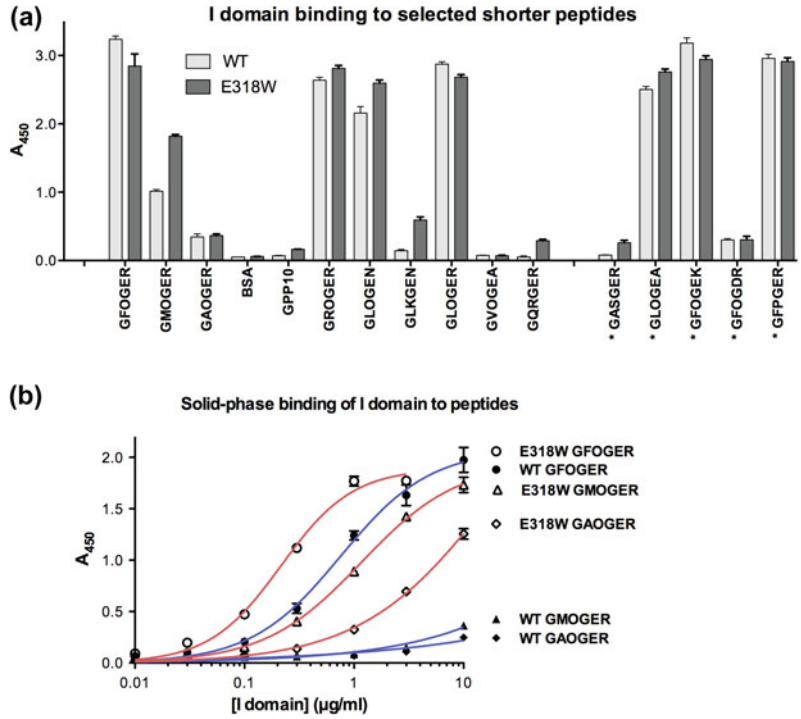

Fig. 9.3 **a** The ability of wild type and E318W α2 I domain to bind to shorter triple-helical peptides. Peptides were presented as in Fig. 9.1, and show that the activating mutation leads to improved binding to the moderate affinity motif, GMOGER, and to some low affinity peptides. **b** Quantitates this effect, and shows that the activating mutation shifts the binding curve threefold or so to the left for GFOGER and GMOGER. For the low affinity GAOGER, binding becomes measurable for E318W compared with wild type α2 I domain. (Reproduced from Carafoli et al. [9] under the creative commons license. See http://creativecommons.org/licenses/by/3.0/)

cells may be activated, perhaps by local cytokine levels in an inflammatory lesion.

9.9 Engineered Collagens as Integrin-Binding Proteins

Finally, we mention the potential for the use of integrin-binding motifs in tissue engineering applications. This concept arose in part from the location of an integrin-binding site in the *Streptococcus pyogenes* adhesin, SCL1, within its 213 residue COL domain [25], subsequently identified as the non-hydroxylated analogue of mammalian binding motifs, GLPGER [10]. Such motifs are rare in bacterial collagens, perhaps unique, although a number of bacteria express collagen-like proteins. Höök's group subsequently designed a sequence, GFPGEN, that was suitable for bacterial expression and that displayed $\alpha 1\beta 1$-selectivity [58]. Bacterial expression of accurately folded collagens becomes a real possibility. The combination of exploration of integrin selectivity with re-engineering into bacterial expression systems makes the production of collagen-like biomaterials by fermentation an exciting prospect.

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Cellular Signaling by Collagen-Binding Integrins

10

Jyrki Heino

Abstract

The four collagen-binding αI domain integrins form their own subgroup among cell adhesion receptors. The signaling functions of $\alpha1\beta1$ and $\alpha2\beta1$ integrins have been analyzed in many experimental models, whereas less studies are available about the more recently found $\alpha10\beta1$ and $\alpha11\beta1$ heterodimers. Interestingly, collagen binding by $\alpha1\beta1$ and $\alpha2\beta1$ often generates opposite cellular responses. For example $\alpha1\beta1$ has often been reported to promote cell proliferation and to suppress collagen synthesis, whereas $\alpha2\beta1$ can in many model systems inhibit growth and promote collagen synthesis. There are obviously cell type dependent factors modifying the signaling. Additionally the structure and the organization of collagenous matrix play a critic role. Many recent studies have also stressed the importance of the crosstalk between the integrins and other cell surface receptors.

Keywords

Integrins · Collagen · Signaling

10.1 Introduction

The members of the collagen receptor subgroup of the integrins recognize their ligands using an inserted domain in their α subunit (αI domain, often called as αA domain). Four heterodimers belong to this category, namely $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ [8, 11, 77, 102]. While $\alpha1\beta1$ and $\alpha2\beta1$ are very abundantly expressed on distinct cell types, the tissue distribution of $\alpha10\beta1$ is mainly limited to cartilage. Integrin $\alpha11\beta1$ is found on mesenchymal cells, e.g. fibroblasts. The four receptors have differences in their ability to recognize extracellular matrix (ECM) and other ligands. Table 10.1 collects the published information about recognition of distinct collagen subtypes by integrins. Furthermore, the fact that the cytoplasmic domains of the α subunits are different [8, 11, 77, 102] suggests that they also generate unique intracellular signals. Numerous cell type and tissue

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Table 10.1 The recognition of distinct collagen subtypes

Fibril-forming collagens (I, II, III, V, XI, XXIV, XXVIII)
Collagens I, II: α1β1/α1I domain, α2β1/α2I domain, α10β1/α10I domain, α11β1/α11I domain
Collagens III, V: α1β1/α1I domain, α2β1/α2I domain, α10β1/α10I domain
Collagen XI: α2I domain [77]
The approximated avidity of α1β1 binding to fibril-forming collagens is lower than that of α2β1. References for α10β1/α10I and α11β1/α11I [110, 76]
Network forming collagens (IV, VIII, X)
Collagen IV: Integrin α1β1/α1I domain, α2β1/α2I domain, α10I domain. Integrin α1β1 seems to prefer type IV over fibril-forming collagens, opposite to α2β1 integrin [47, 76]
Collagen VIII: Platelet binding is mediated by α2β1 [77]
Collagen X: α2β1 [59]
Beaded-filaments forming collagen (VI)
Collagen VI: α1β1/α1I, α10I domain. Binding by α2β1/α2I domain is much weaker [96]
Anchoring fibrils forming collagen (VII)
Collagen VII: NC1 domain in is recognized by α2β1 on human fibroblasts [15]. Platelet binding is mediated by α2β1 [77]
FACIT collagens (IX, XII, XIV, XVI, XIX, XX, XXI, XXII)
Collagen IX: α1β1/α1I domain, α2β1/α2I domain, α10β1/α10 I domain, α11I domain [46]
Collagen XIV: CD44, unlike α1β1 or α2β1 [26, 48]
Collagen XVI: α1β1/α1I domain, α2β1/α2I domain. Binging by α1β1 is stronger [25]
Transmembrane collagens (XIII, XVII, XXIII, XXV)
Collagen XIII: α1β1 integrin/α1I domain. Binding by α2β1/α2I domain much weaker [69]
Collagen XVII: The largest collagenous domain (COL 15) cannot be recognized by the collagen receptors. However, when denatured the multiple KGD motifs can be used by α5β1 and αV-integrins [70]
Collagen XXIII: α2β1 [76]
Multiplexins (XV, XVIII)
Collagen XVIII: α1β1 [24]. Endostatin, the C-terminal cleavage product is recognized by α5β1 and αV-integrins [76]

(Note that here collagens XXVI and XXVIII have not been listed to any of the subgroups)

specific factors, e.g. interplay with other cellular receptors, also modify the signaling by the collagen receptors [42].

This chapter is focused on the four members of the collagen receptor subgroup of the integrins. However, also other integrins have been reported to function as collagen receptors. Integrin α3β1, generally known as a laminin receptor, may also act as an assisting collagen IV receptor [23]. Similarly, the leukocyte αI domain integrins can bind to various collagen subtypes and they can be considered as low-avidity or assisting collagen receptors [54]. Furthermore, denatured collagen (e.g. collagen I) can be recognized by the fibronectin receptor integrins based on the cryptic RGD motifs in collagen α chains [34].

10.2 Collagens and Other ECM Ligands

Collagens are structural proteins of extracellular matrix that typically have triple helical domains of variable length [76]. Collagens form, for example, large fibrils in connective tissues and networks in basement membranes, while some collagens are transmembrane proteins. Metazoans from sea sponges [2] to mammals express collagens and in man, 28 structurally and functionally different collagen subtypes have been published [76]. The collagen subtypes are named from I to XXVIII based on the order in which they have been found. The collagen family is composed of several subgroups. The fibril-forming

collagens have a long, continuous triple helix that gives to the molecule a rigid, rod-like structure. These collagens form large fibrils, which is essential for the structural integrity and the tensile strength of the tissues. The network-forming collagens have interruptions in the triple helix. Basement membrane collagen IV belongs to this subfamily. Two collagen subtypes have unique functions and they are the only members of the corresponding subgroups: beaded-filaments are built from collagen VI and anchoring fibrils from collagen VII. Fibril-associated collagens with interruptions in triple-helices (FACIT) form a large subgroup. Collagens IX and XII are typical FACITs. All FACIT collagens may not, however, be able to bind to fibrils. Collagens XIII and XVII were the first subtypes shown to be transmembrane proteins. Collagen XVII is a structural component of hemidesmosomes, whereas collagen XIII is found, for example, in muscle, bone and skin. Multiplexins are collagens that are associated to basement membranes. Collagens XV and XVIII belong to this subfamily. Their C-terminal cleavage products have become known as angiogenesis-blocking endostatins [71].

Specific collagenous motifs are recognized by integrin αI domains. Best known is the GFOGER (O = hydroxyproline) sequence in triple-helical conformation [52], which is a binding site for all four collagen receptor integrins. GLOGER, GASGER, GROGER, and GLOGEN represent other similar motifs [58]. However, many collagen subtypes are not homotrimers, but the triple helix is formed by two or three different α chains. In these cases the integrin binding mechanism may be different. For example in collagen IV α1β1 integrin may recognize one arginine and two aspartic acid residues all coming from a different α chain [76]. Many reports have named the fibril-forming collagens as high-avidity ligands of α2β1 and α11β1 integrins, whereas α1β1 seems to the best receptor for collagens IV and XIII [35, Table 1]. Receptor for collagen XIV is CD44 and it may not be an optimal ligand for the integrins [48]. Recognition of distinct collagen subtypes may also be dependent on the activation stage of the integrin [77, 12]. The αI domain has at least two activation stages. In nonactivated integrins the αI domain is in the closed conformation, that is able to recognize the ligands, but the interaction is weaker than with the activated, open αI domain. Activation may also diminish selectivity between high and low avidity binding motifs [12] and ligand proteins [77] and between hetero and homotrimeric collagen subtypes [12].

The collagen receptors also have non-collagenous ligands. Integrin α1β1 is a receptor for laminins, collagen IV derived antiangiogenic degradation product called arresten [77] and semaphorin protein, Sema7A [72].

Integrin α2β1 has numerous ligands, including ECM proteins tenascin C [76] and chondroadherin [10] as well as different laminins. This receptor can also bind to proteoglycans, such as decorin [32] and endorepellin (C-terminal domain of perlecan; [6]), and collectin family members, namely C1q complement protein, mannose-binding lectin (MBL) and surfactant protein A (SP-A) [77].

10.3 Regulation of Cellular Signaling Pathways by the Collagen Receptor Integrins

During the canonical integrin outside-in signaling, the ligand binding induces rapid increase in the levels of phosphatidylinositol-4,5-bidphosphate and phosphatidylinositol-3,4,5-triphosphate and promotes the tyrosine phosphorylation of proteins such as focal adhesion kinase (FAK), p130Cas and Src [57]. Soon after that small GTPases belonging to the Rho-family are activated [57]. Finally, the integrins regulate many pathways controlling cell survival, proliferation, differentiation, migration and metabolism. There is no reason to believe that the collagen receptors would act in a different manner. Indeed, there are numerous papers using different experimental models and demonstrating the regulation of FAK, Src, p130Cas, mitogen activated protein kinases (MAPKs), including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, as well as phosphoinositide

3-kinase (PI3K) and Akt (Protein kinase B) by $\alpha1\beta1$ and $\alpha2\beta1$ integrins. Activation of Rac-1 GTPase has been connected to $\alpha1\beta1$ mediated cell migration in vitro [76] and invasion in vivo [77]. Integrin $\alpha2\beta1$ has been reported to activate RhoA and slow down cell locomotion [77]. Integrin $\alpha2\beta1$ may also regulate Rac-1 by orchestrating the membrane anchorage of Rac [9].

The functions of $\alpha2\beta1$ have been investigated in detail in platelets. These studies have revealed that Rac and/or p21-activated kinase (PAK) activation, but not Rho, participate in integrin $\alpha2\beta1$ mediated platelet spreading on collagen and that Src family kinases and PI3K are also involved [76]. Another study indicates that Pyk2 (a FAK-family protein tyrosine kinase) regulates PI3K and Akt downstream of integrin $\alpha2\beta1$ [20]. In platelets $\alpha2\beta1$ has also been connected to the regulation of Spleen tyrosine kinase (Syk), SH2 domain-containing leukocyte phosphoprotein (SLP-76), and phospholipase C$\gamma2$ (LPC$\gamma2$) as well as plasma membrane calcium ATPase and FAK [33, 39]. Many reports indicate that both $\alpha1\beta1$ [17, 18] and $\alpha2\beta1$ [38] can regulate the formation reactive oxygen species (ROS), which partially explains their effects on cellular functions, e.g. p38 MAPK phosphorylation and cyclin expression [38].

The general signaling events are often mediated by the β subunit and therefore they are not specific to any individual $\beta1$ containing heterodimer. Still, the collagen receptors may have different, even opposite, effects on gene expression. For example, $\alpha1\beta1$ is a negative regulator of collagen I synthesis [55, 77], whereas $\alpha2\beta1$ increases the expression [40, 77]. Some regulatory functions of the collagen receptors seem to be dependent on the cytoplasmic tails of the α subunits. For example the effect of $\alpha1\beta1$ on Rac-1 activation and consequently on cell migration is dependent on the $\alpha1$ cytoplasmic domain [76]. The mechanism of signaling through $\alpha2\beta1$ integrin has also been studied by deletions, mutations and swaps of the $\alpha2$ subunit cytoplasmic domain [13, 40, 45, 49, 51]. Deletion of the entire tail targets the integrin to the focal adhesion sites, even in the absence of collagen [45]. The essential role of the $\alpha2$ cytoplasmic tail in other functions of $\alpha2\beta1$ integrin, such as contraction of collagen gels, has also been well characterized [13]. Replacement of the $\alpha2$ cytoplasmic tail with one from the $\alpha1$ integrin renders the integrin unable to signal normally [40, 49, 51]. This also supports the idea that different collagen receptors have distinct signaling functions. Furthermore, these observations suggest that the cytoplasmic domains of the collagen receptor α subunits may be directly connected to cellular signaling proteins, or that they modify the molecular interactions of the $\beta1$ cytoplasmic domain. Still very little is known about α subunit binding proteins or the mechanisms of α specific signaling. The cytoplasmic tail of $\alpha1$ integrin selectively interacts with a ubiquitously expressed protein tyrosine phosphatase TCPTP (T-cell protein tyrosine phosphatase) and activates it after cell adhesion to collagen [61]. Other α subunit binding proteins include Rab21 and SHARPIN, but they are not selective for any single α subunit. Rab21 regulates integrin trafficking [73] and SHARPIN is an inhibitor of integrin ligand binding function [76].

Many integrins may also act as cellular receptors for viruses. Human pathogen, echovirus-1 (EV-1) binds to the $\alpha2$I domain in $\alpha2\beta1$ integrin and is rapidly internalized into the host cell. Interestingly, EV-1 seems to recognize the closed conformation of $\alpha2$I domain and keep the integrin in the nonactivated stage [43]. Still the virus is able to activate protein kinase C-α (PKC-α) and Rac-1, which is also requited for the macropinocytosis-like entry of the virus-integrin complex [44, 77]. In this case the clustering of integrins seems to be the activating factor rather than the conformational change in the receptors. The nonactivated stage of $\alpha2\beta1$ may also have other biological functions, e.g. during platelet collagen binding under shear stress [67]. Actually, activated $\alpha2\beta1$ integrins are very rapidly internalized in a ligand dependent manner, which leads to remarkable decrease in the number of collagen receptors on platelets [76].

10.4 Interplay of the Collagen Receptors with Growth Factor Receptors

Recent studies have indicated that integrins have numerous mechanisms to interact with growth factor receptors [42]. Cell adhesion is often required for the establishment of molecular platforms that enable the signaling by growth factor receptors. Integrins may even activate the growth factor receptors in the absence of the growth factor. Integrins can also orchestrate the trafficking of other receptors and in that way regulate the copy number of growth factor receptors on cell surface [42].

The interplay between collagen receptors and epidermal growth factor receptor (EGFR) has been studied in different cellular model systems. Integrin $\alpha1\beta1$ is reported to negatively regulate EGFR. This may be related to the ability of $\alpha1\beta1$ to increase caveolin-1 levels and to activate protein tyrosine phosphatase TCPTP [18, 61]. Integrin $\alpha2\beta1$ is known to modify EGFR signaling, too. EGFR may also reduce the levels of $\alpha2\beta1$ on cell surface by increasing its internalization [66]. Published findings have also suggested that crosstalk between hepatocyte growth factor receptor (HGFR/c-met) and $\alpha2\beta1$ integrin is requited for mast-cell activation [62].

In addition to ECM proteins many integrin can also directly bind to growth factors [42]. Accordingly, integrin $\alpha1\beta1$ is not only a receptor for collagens, but it has also been reported to recognize a semaphorin protein, Sema7A, that enhances axon growth [72]. Sema7A binding to $\alpha1\beta1$ integrin can activate several signaling proteins, including FAK, ERK MAPKs [72], Abelson (Abl), and Abl-related gene (Arg) tyrosine kinases [64].

10.5 Crosstalk Between Integrins and Discoidin Domain Type Collagen Receptors

The collagen receptor integrins are not the only cellular receptors that can recognize collagenous triple-helical motifs [58]. Platelet glycoprotein VI (GPVI) on platelets is critical for response to collagen. Discoidin domain receptors 1 and 2 (DDR1 and DDR2) are tyrosine kinases that bind to collagens and regulate e.g. cell proliferation. Leukocyte associated immunoglobulin-like receptor 1 (LAIR-1) is an inhibitory receptor on leukocytes. The triple helical motifs formed by peptides harboring GVMGFO sequences have been described as binding sites for DDR1 and DDR2. The minimum functional binding site for GPVI contains two GPO triplets in collagenous triple helix. Similarly, LAIR-1 binds to peptides containing multiple GPOs [58]. Thus, these receptors do not compete with the integrins in collagen binding.

Activation and autophosphorylation of DDRs are independent of the integrins [76]. However, several studies indicate that the signaling functions of DDRs and collagen receptor integrins are linked together. DDRs can for example regulate integrin activity [1, 77]. The interplay between the two receptor systems is also obvious in studies focused on collagen I induced epithelial-mesenchymal-transition in pancreatic cancer cells. During the process N-cadherin is upregulated by JNK-dependent mechanism. Both receptors are needed for p130Cas-dependent activation of Rap1, but they act in a different manner. DDR1 regulates Pyk2, while $\alpha2\beta1$ integrin activates FAK [76].

In another study DDR1 was shown to inhibit collagen-induced tyrosine phosphorylation of Stat 1/3 and cell migration triggered by $\alpha2\beta1$ integrin via SHP-2. SHP-2 is a phosphotyrosol phosphatase (PTP) that via SH2 domain binds to phosphorylated tyrosine residues in DDR1 [77].

10.6 Regulation of Cell Proliferation and Survival by the Collagen Receptors

Analysis of $\alpha1$ integrin deficient knock-out mice has supported the idea that $\alpha1$ might promote cell proliferation, since the dermis of the animals seems to be hypocellular [50] and their bone marrow derived mesenchymal stem cells are less

proliferative than those of the control animals [27]. Integrin $\alpha1\beta1$ is among the integrins that can activate Shc (a SH2 domain containing adaptor protein) in a process requiring caveolin-1 and Fyn (a protein tyrosine kinase) [50, 76]. Shc activation subsequently leads to activation of Ras and the growth promoting MAPKs. However, the fact that $\alpha1\beta1$ negatively regulates EGFR [61] and caveolin-1 phosphorylation [7] also connects this integrin to growth inhibiting mechanisms. The idea that $\alpha1\beta1$ can generate different or even opposite signals is also supported by the observation that the binding of this receptor to two different domains in laminin has distinct effects [22].

Integrin $\alpha2$ null mice are viable, fertile and without defects that could suggest general dysregulation of cell growth [14, 37]. However, in cell culture assays $\alpha2\beta1$ is capable of generating negative growth signals. Its interaction with laminin, a low affinity ligand, results in growth arrest in endothelial cells [63]. It also increases cell commitment toward quiesence by a mechanism involving changes in the anchorage of Ras to membranes and a tetraspanin CD9 [9]. The role of $\alpha2\beta1$ as a negative growth regulator is also in agreement with the observation that the expression level of $\alpha2$ is often very low in breast cancer cells [77]. The effect of collagen—$\alpha2\beta1$ interaction on cell proliferation seems to be dependent on the organization of the collagenous matrix. In mesenchymal cells, including smooth muscle cells and fibroblasts, fibrillar collagen prevents proliferation [28, 53]. Similar results have been reported with melanoma cells [36]. In fibroblasts growth arrest may require that the cells are inside floating and contracting collagen gels [28]. In smooth muscle and melanoma cells as well as in fibroblasts, growth arrest has been connected to $\alpha2\beta1$ function and the accumulation of cyclin/cyclin-dependent kinase inhibitor, p27kip [28, 36, 53]. Recent studies have also indicated that the crosslinking and therefore the stiffness of collagenous matrix is an important regulator of cell behavior [56].

On a contrary in murine mammary gland-derived epithelial cells, $\alpha2\beta1$ has been reported to increase proliferation, when tested in monolayer cultures on non-fibrillar collagen [76]. In these cells the intracellular tail of $\alpha2$ subunit has been analyzed by targeted mutations, and two distinct sites have been identified which regulate p38 and ERK pathways [49, 51], connected to cell migration and proliferation, respectively. Similarly, in human adenocarcinoma cells (Caco-2) interaction of $\alpha2\beta1$ with collagen IV promotes G1/S transition [38].

The conclusion is that the organization of the collagenous matrix is critical for the action of $\alpha2\beta1$. It has been speculated that the clustering of $\alpha2\beta1$ by antibodies or non-fibrillar collagen may actually promote proliferation, while fibrillar collagen prevents $\alpha2\beta1$ clustering and therefore inhibits proliferation [36]. Similarly, physical forces, such as the stiffness of the tissue [56] or the shear stress in blood stream [67], may be important modulators of integrin action and signal transduction.

A recent study indicates that integrins $\alpha2\beta1$ and $\alpha11\beta1$ promote the survival of mesenchymal stem cells [74]. Integrin $\alpha2\beta1$ is also known to be a marker protein of prostate epithelial stem cells [19]. Anti-apoptotic effect of $\alpha2\beta1$ has been reported with human mammary epithelial cells [4], Jurkat T cells [3], A431 cells [77] and Madin-Darby canine kidney cells [76]. In an acute liver injury model in mice $\alpha1\beta1$ has been shown to mediate survival promoting signals after contact to collagen XVIII [24].

In certain experimental models the collagen receptors also promote apoptosis. Release of mechanical tension in a three-dimensional collagen gel model triggers apoptosis in fibroblasts [31, 65]. In these conditions $\alpha2\beta1$ mediates cell adhesion to collagen and is essential for contraction. Function blocking antibodies against $\alpha2\beta1$ and $\alpha1\beta1$ integrin can reduce the number of apoptotic cells, and $\alpha2$-negative rhabdomyosarcoma cells undergo apoptosis only if they are cDNA-transfected to express the $\alpha2$ subunit [65].

Apoptosis may be partially regulated by the same signaling pathways as proliferation. In addition to the ERK pathway, Akt seems to be involved. In fibroblasts and osteosarcoma cells, protein phosphatase 2A (PP2A) is activated in

a process that requires the presence of α2 cytoplasmic domain and Cdc42 activity [41]. Activation of PP2A leads consequently to dephosphorylation of Akt, a well-known promoter of cell survival. PP2A can also inhibit the cell cycle in several different ways, and α2β1 dependent activation of PP2A may be one mechanism leading to growth arrest. Dephosphorylation of Akt inside collagen gel can be prevented by α2 integrin antibodies [41]. Another study indicates that apoptosis of fibroblasts inside contracting collagen gels can be prevented by β1 integrin antibodies which also prevent Akt dephosphorylation [76].

Integrin α1β1 is a receptor for arresten, a 26 kDa non-collagenous domain of α1-chain in collagen IV. Arresten promotes apoptosis of endothelial by decreasing the amount of anti-apoptotic molecules of the Bcl-family, namely Bcl-2 and Bcl-xL [68].

10.7 Regulation of Matrix Gene Expression by the Collagen Receptors

Several lines of evidence support the idea that α1β1 integrin is a negative regulator of collagen synthesis, especially in cells that are surrounded by three-dimensional collagenous matrix. Early observations using osteosarcoma cells with a low expression level of α1β1 showed that these cells do not down-regulate collagen synthesis inside collagen [77]. This was later confirmed by experiments performed with cells derived from α1 integrin deficient mice [29]. Furthermore, experiments utilizing functional integrin antibodies or mutant α1 integrins have led to the same conclusion [55, 77]. Similar regulatory mechanisms may also function in tissues since α1 knock-out mice have increased collagen synthesis rate in their dermis while a concomitant increase in matrix metalloproteinase (MMP) expression prevents the accumulation of collagen [29]. Integrin α1 null mice are also more sensitive to glomerulosclerosis than their wild type littermates [16].

Overexpression of α2β1 integrin in cells increases collagen synthesis, suggesting that α2β1 is a positive regulator of collagen gene expression [40, 77]. Increased collagen synthesis can be prevented using selective inhibitors of the α isoform of p38 MAPK. p38 is activated by α2β1 after contact with collagen [40]. Activation of the p38 pathway has been frequently observed after α2β1—collagen interaction in several different cell lines and experiment models [40, 49, 51, 77, 76]. The mechanism of collagen gene suppression by α1β1 is not clear but the receptor can activate ERK [50] that is known to be a negative regulator of collagen synthesis [77]. It is also possible to speculate that the opposite effects of α1β1 and α2β1 on many cellular functions may partially be due to the alterations in the balance between p38 and ERK pathways.

Cell contact to three-dimensional collagen activates the expression of collagenase-1 (matrix metalloproteinase-1, MMP-1) [30]. This phenomenon has been linked to signaling through the α2β1 integrin [77]. Other MMPs regulated by either α1β1 or α2β1 include stromalysin-1 (MMP-3) [60] and collagenase-3 (MMP-13) [77]. In skin fibroblasts inside collagen collagenase-1 (MMP-1) expression seems to be activated by a pathway involving PKC-ζ and nuclear factor κB (NF-κB) [76, 77]. However, the above described p38 pathway may also participate in the process [76]. Interestingly, α2β1 can also regulate its own expression by a positive signaling loop involving PKC-ζ/NF-κB [77]. The p38 pathway seems to mediate the upregulation of MMP-13 by α2β1 integrin [77].

Signaling by α11β1 integrin has recently been shown to induce MMP-13 expression [5]. Integrin α1β1 has been reported to regulate MMP-2, MMP-9 and MMP-14 in a p38-dependent manner in mesengial cells [21] and MMP-2 and MMP-9 expression in colon cancer cells via p130Cas and JNK [77]. In general, there is a strong link between the collagen receptor integrins and the expression of MMPs. This connection may play an important role in the maintenance of tissue homeostasis, in the regulation of wound healing and tissue repair processes and during cell invasion.

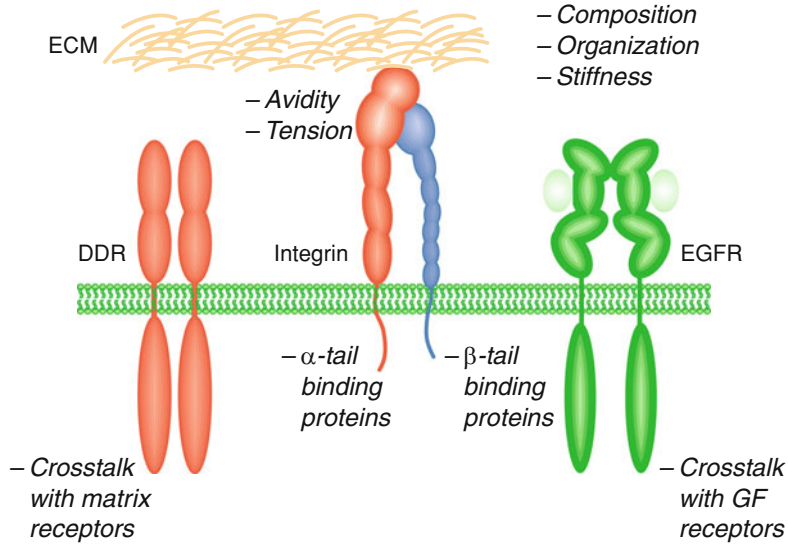

Fig. 10.1 Numerous factors influence on the cellular signals generated by the collagen receptor integrins. Composition, organization and stiffness of collagenous ECM are all important factors. Activation stage of integrins regulates the avidity and specificity of ligand binding. Crosstalk with other adhesion and growth factor receptors is involved. There are also cell type specific differences in the expression of integrin cytoplasmic domain binding proteins

10.8 Perspectives

Cellular signaling after adhesion to collagen is influenced by numerous factors (Fig. 10.1). Different collagen subtypes are recognized by different receptors and also the organization of the ECM is critical. Recent observations have also stressed the important role of ECM stiffness. Activation stage of integrins regulates the avidity and specificity of ligand binding. Integrin signaling is also influenced by other adhesion receptors, e.g. DDR-type collagen receptors, and growth factor receptors. Finally, cell type specific differences, e.g. in the expression of integrin cytoplasmic domain binding proteins, may modify the activation of distinct cellular pathways.

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The Therapeutic Potential of I-Domain Integrins

11

Marian Brennan and Dermot Cox

Abstract

Due to their role in processes central to cancer and autoimmune disease I-domain integrins are an attractive drug target. Both antibodies and small molecule antagonists have been discovered and tested in the clinic. Much of the effort has focused on $\alpha L\beta 2$ antagonists. Maybe the most successful was the monoclonal antibody efalizumab, which was approved for the treatment of psoriasis but subsequently withdrawn from the market due to the occurrence of a serious adverse effect (progressive multifocal leukoencephalopathy). Other monoclonal antibodies were tested for the treatment of reperfusion injury, post-myocardial infarction, but failed to progress due to lack of efficacy. New potent small molecule inhibitors of αv integrins are promising reagents for treating fibrotic disease. Small molecule inhibitors targeting collagen-binding integrins have been discovered and future work will focus on identifying molecules selectively targeting each of the collagen receptors and identifying appropriate target diseases for future clinical studies.

Keywords

I-domain integrins · Therapeutics · Integrin antagonists · Integrin structure

11.1 Introduction

The discovery of the first integrin [129] and the realization that this was only a member of a large family of cell adhesion molecules [108] opened up possibilities for novel therapeutics for diseases such as cancer, inflammation and thrombosis. Prior to this, drug discovery was very much chemistry-led where novel chemicals were screened for potential biological activities.

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The discovery of the integrin provided an opportunity for target-led drug discovery where chemicals could be screened for their ability to bind to specific receptors. The potential for this new paradigm was confirmed with the approval of the αIIbβ3 antagonist abciximab in 1993. However, since then, aside from two other αIIbβ3 antagonists the only other integrins with approved antagonists are the α4 integrins.

Why the poor success in developing anti-integrin agents? It is certainly not due to the lack of a clinical potential for integrin antagonists as integrins are clearly involved in many of the big diseases such as cardiovascular disease, autoimmune disease and cancer. It is not due to a difficulty in discovering potent antagonists as many antibody and small molecule antagonists have been discovered. Most of the difficulties have arisen due to the complexity of integrins and a poor understanding of the pharmacology of these agents. Despite the poor record of developing anti-integrin agents there is still great potential for the development of this class of drugs. This chapter focuses on the history of integrin antagonists with a specific focus on the development of I-domain antagonists.

11.2 Integrin Families

Integrins are cell adhesion molecules that are found on virtually every cell in the body where they mediate cell–cell and cell-substrate interactions, which are essential for regulating cell growth and cell function. However, they do not simply act as "glue-like" molecules as they are true receptors, generating intracellular signals. Their importance is reflected in the diverse range of diseases in which integrins play key roles including cancer, thrombosis, autoimmune diseases and infection.

Integrins are heterodimers formed from the combination of an α and a β subunit. As there are only eight distinct β subunits and eighteen α subunits combining to form twenty-four unique receptors many β subunits must complex with more than one α subunit (see Preface). Since there are more α subunits than β subunits the integrins were originally described as a superfamily composed of families defined by their β subunit. Table 1 in the Preface chapter lists the integrin families as defined by their β subunits and their associated ligands.

Initially defining integrin families based on their β subunit made sense, as it appeared that the α subunits only associated with a specific β subunit. However, it soon became clear that this is not so as αV can associate with many different β subunits and the α subunit appears to be important in defining the ligand binding properties of an integrin. Thus, among the twelve β1 integrins there is a wide range of ligands with little in common between them other than their shared β subunit. On the other hand, all five αV integrins have similar ligand-binding properties and all bind vitronectin. The β2 integrins are unique in that they do not share their α subunits with any other β subunit and their ligand-binding properties are very similar. Integrins can also be categorized according to their amino acid recognition sequences. The most common recognition sequence is Arg-Gly-Asp (RGD), which is recognized by many integrins. However, this is complicated by the fact that some integrins can bind some proteins in an RGD-dependent manner and others in an RGD-independent manner. For instance αIIbβ3 can bind to fibrinogen via the 2-RGD sequences and via the γ-chain dodecapeptide [123]. As a result defining integrin families requires a more flexible system. Integrins can also be classified by structure. In particular 9 integrin α-subunits contain an I-domain, which is important for ligand binding (see Fig. 11.1). Thus, integrins can be defined as I-domain and non-I-domain-containing integrins. This review will focus on the I-domain-containing integrins and their properties are described below after being loosely grouped into families based on their ligand-binding properties and α and β-subunits.

11.2.1 β2 Integrins

The β2 integrins are a well-defined and distinct group of integrins and their α subunits exclusively associate with the β2 subunit. They are

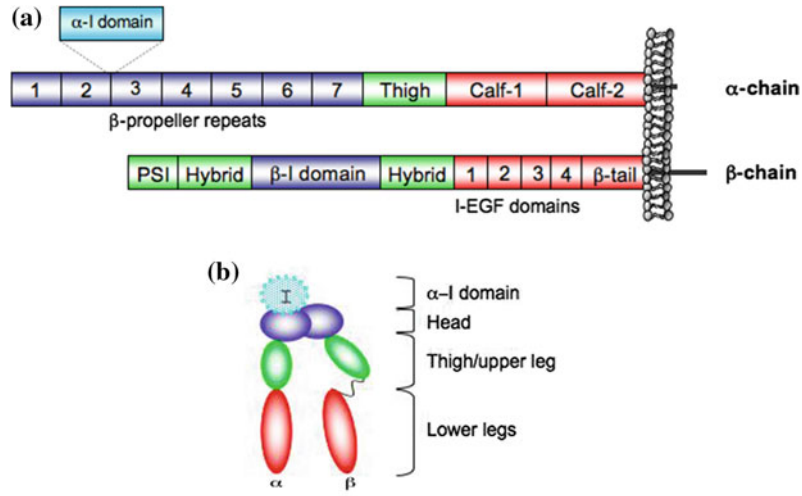


Fig. 11.1 Schematic of the domain structure of I-domain integrins [25, 119, 148]. **a** The I-domain is inserted between the 2nd and 3rd beta propeller subunit on the alpha chain. **b** Outline of the domain structure of an activated I-domain containing integrin

primarily found on leucocytes and are important in normal immune function. The $\beta 2$ subunit was originally identified as CD18 and the α subunit as CD11. There are four α subunits: αL (LFA-1), αM (Mac-1), αX and αD. All four receptors bind at least one member of the ICAM family, two are complement receptors ($\alpha M\beta 2$ (CR3) and $\alpha X\beta 2$ (CR4)) and two are fibrinogen receptors ($\alpha X\beta 2$ and $\alpha D\beta 2$).

11.2.2 Collagen-Binding Integrins

There are 5 integrins that bind collagen and all are $\beta 1$ integrins ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 10$ and $\alpha 11$) with differing collagen selectivity [154] (see Preface chapter).All except $\alpha 3\beta 1$ are I-domain containing integrins. $\alpha 3\beta 1$ is a high-affinity receptor for laminin but it also binds collagen type IV and VI through the collagen NC1 domain [2, 11, 16], which is distinct to the binding of the other collagen receptors. $\alpha 1\beta 1$ is found on many cell types including endothelial cells, fibroblasts, astrocytes, T-cells, natural killer cells and macrophages not B-cells. Expression levels usually increase with cytokine stimulation. $\alpha 2\beta 1$ is expressed on platelets, epithelial cells, and many mesenchymal cell types. $\alpha 10\beta 1$ is expressed on chondrocytes [19] while $\alpha 11\beta 1$ is expressed on fibroblasts [100].

11.2.3 $\alpha E\beta 7$

The $\beta 7$ integrins are found on lymphocytes. $\alpha E\beta 7$ is the E-Cadherin receptor primarily found on intraepithelial T-lymphocytes while $\alpha 4\beta 7$ is found on lymphocytes in gut-associated lymphoid tissue. $\alpha E\beta 7$ facilitates lymphocyte homing to the lamina propria resulting in increased expression of $\alpha E\beta 7$, which facilitates lymphocyte extravasation [41]. It is found on T-lymphocytes especially in the gut and in high potency regulatory T-cells [73].

11.3 Integrin Ligands

Integrins bind to a diverse collection of ligands that are large molecules. They are either subendothelial matrix proteins such as fibronectin, vitronectin and collagens or plasma proteins such as complement factors, C-reactive protein [14] and fibrinogen. There is also a group of secreted proteins known as small integrin-binding ligand N-linked glycoproteins (SIBLINGs), which include osteopontin and bone sialoprotein [8]. An interesting ligand for a number of integrins is latent TGF$\beta 1$ (LAP-TGF-β). This is an inactive complex of TGF that requires activation by binding to integrins before it becomes biologically active [91].

Many integrins recognize the amino acid sequence Arg-Gly-Asp (RGD) in their ligands. However, RGD is a relatively common motif in proteins many of which are not known to be integrin ligands. Also many RGD-containing proteins can be shown to bind to an integrin that they are unlikely to encounter in vivo or they may require unnatural conformations to expose the RGD motif and thus are not genuine ligands. NGR is also another integrin-recognition motif used by some integrins [68].

11.4 Integrin Physiology

Cell–cell and cell-substrate interactions are critical for every cell of the body, even those in the circulation. Contact with other cells and extracellular matrix components regulates the activity of all cells and since integrins are an important family of receptors that mediate these interactions, they play essential roles in the function of most cells in the body. Areas where integrins are especially important are those that involve growth of tissue or where cell attachment is necessary for function. Thus, embryonic development and the growth of new blood vessels (angiogenesis) [121] are critically dependent on integrins as is the immune system [122] where immune cell attachment is necessary for normal function.

11.5 Integrin Pathology

While integrins have many different physiological roles, identifying a role for integrins in pathology, especially with respect to identifying drug targets, is difficult. This is because many diseases are multi-factorial and while integrins may play a role in the disease process they are only one of many receptors involved. Many cells have multiple integrins with similar binding properties, which can compensate for the inhibited integrin and as a result targeting specific integrins often does not provide therapeutic benefit despite a role for that integrin in the disease process.

11.5.1 Thrombosis

The first disease to be clearly identified with integrins was thrombosis and this was the first therapeutic target for anti-integrin therapy. Thrombosis occurs when platelets adhere to damaged blood vessels and become activated. These activated platelets recruit other platelets resulting in the formation of a haemostatic plug. This is the essential mechanism for preventing blood loss but inappropriate thrombus formation can lead to a stroke or myocardial infarction.

One of the earliest events in haemostasis is the interaction between platelets and exposed collagen in the damaged endothelium. While there are 5 collagen-binding integrins only one ($\alpha2\beta1$) is expressed on the platelet. The $\alpha2\beta1$ interaction with exposed collagen in conjunction with a second, non-integrin collagen receptor (GPVI) leads to platelet activation [23]. The platelet-platelet interaction that mediates thrombus formation is facilitated by fibrinogen binding to the integrin αIIb$\beta3$, which becomes activated as a result of platelet activation. Currently the only approved anti-integrin inhibitors for thrombosis are the αIIb$\beta3$ antagonists although there are also inhibitors of $\alpha2\beta1$ under development [84].

11.5.2 Cancer

During carcinogenesis the growth of the tumour and its subsequent metastasis is highly dependent on the cell being able to regulate its attachment to the extracellular matrix and adjacent cells. As integrins play an important role in cell attachment the ability to up-regulate and down-regulate these receptors is critical in carcinogenesis [90]. Integrins play important roles in cell attachment, survival, migration and invasion [59]. Integrins are also essential in the process of angiogenesis, which is also critical for cell growth [6, 121]. As a result there has been a focus on inhibiting integrins to disrupt tumour growth, cancer-associated fibroblasts and metastasis [30].

Arresten is an angiogenesis inhibitor that is derived from collagen. It binds to $\alpha1\beta1$ and inhibits invasion of a squamous cell carcinoma in vitro and in vivo [3]. Endorepellin is a anti-angiogenic fragment of perlecan [87] and binds to both $\alpha2\beta1$ and VEGF receptor triggering their down regulation [50]. $\alpha1\beta1$ is implicated in peritoneal dissemination of gastric cancer cells [44]. $\alpha2\beta1$ acts to inhibit metastasis in mouse models of cancer [103].

11.5.3 Immunology

Both $\beta1$ and $\beta2$ integrins are important in immune function where they play an essential role in localizing the immune response to the site of inflammation. Engagement of the T-cell receptor and subsequent inside-out signalling leads to activation of T-cell integrins [122]. While inhibition of these integrins can modulate the immune response the specific integrin to target or immune disorder to treat have yet to be determined.

Much of the work has focused on the role of $\alpha4$, which is implicated in multiple sclerosis [9, 47, 106, 126] and Crohn's disease [131]. Both $\alpha4\beta1$ and $\alpha L\beta2$ mediate leucocyte adhesion in an animal model of epilepsy and anti-$\alpha4$ antibodies reduced seizure activity [39]. $\alpha L\beta2$ antagonists have shown potential benefit in lupus [135], renal transplant [138], psoriasis [34, 75] and experimental autoimmune encephalomyelitis [139]. $\alpha2\beta1$ is important co-stimulatory molecule on Th17 cells [10].

11.5.4 Infection

A number of infectious agents have developed the ability to interact with integrins and subsequently become internalized allowing access to the intracellular milieu. There are three general mechanisms used to achieve this: binding of integrin ligands that mediate the interaction, a direct interaction with the integrin or binding of a secreted product. The $\alpha M\beta2$ and $\alpha4\beta1$ ligand mindin [58] also acts as a pattern recognition molecule for microbes and thus plays a role in both adaptive and innate immunity [76]. $\alpha M\beta2$ and $\alpha X\beta2$ are complement receptors [83, 111].

Streptococcus agalactiae and *Staphylococcus aureus* have both been shown to bind osteopontin, which triggers phagocytosis by binding to monocyte $\alpha X\beta2$ in an RGD-independent manner [113]. *Shigella flexneri* interacts directly with integrin-linked kinase to enhance adhesion to cells [66].

LFA-1 binds RTX (repeat in toxin) family of cytotoxins from a number of different species [28, 31, 89]. *Helicobacter pylori* Vac A toxin binds to $\beta2$ integrins enabling it to enter T-lymphocytes [115]. Peptides derived from $\beta2$ integrin bind LPS and were found to reduce mortality in a mouse model of sepsis [145]. Viruses have also been shown to bind to integrins, which can facilitate cell entry. Rotavirus binds to $\alpha2\beta1$ and $\alpha4\beta1$ [43, 51, 52]. The interactions of viruses with integrins has been reviewed by Stewart and Nemerow [127].

11.5.5 Osteoporosis and Kidney Disease

Osteoporosis occurs when the balance between bone formation and degradation is disturbed. Integrins play an important role in the function of osteoclasts, which are responsible for degradation of bone. Osteoclast $\alpha1\beta1$ is responsible for adhesion of osteoclasts to collagen and polymorphisms in this receptor are related to bone mineral density and fractures [71]. Integrin $\alpha1$ expression is increased in mesangial cells in Alport disease [125]. $\alpha1\beta1$ is involved in diabetic neuropathy [152]. $\alpha1\beta1$ binding to collagen IV down-regulates collagen IV synthesis highlighting the importance of this integrin for basement membrane dynamics [116].

11.6 Structural Analysis of I-Domain Integrins

I-domain integrins are a subgroup of integrins containing an inserted I-domain between the β propeller domains 2 and 3 on the α subunit

(Fig. 11.1). I-domain containing integrins bind to their ligands through the metal ion-dependent adhesion site (MIDAS) on their I-domain. Collagen is represented bound to α2 as an example of this mechanism of action in Fig. 11.2. Ligand binding leads to integrin activation through movements of the α1 and α7 helices in the I-domain, which translate to allosteric movements in the integrin and subsequent signaling events. The design of integrin antagonists has presented some difficulties as competitive antagonists often lead to allosteric changes and therefore act as partial agonists. I-domain containing integrins also have a second I-like domain on their β chains. With activation, an interaction occurs between the two I-domains leading to signaling events. This has led to the development of inhibitors to the linker site (Fig. 11.3) [118].

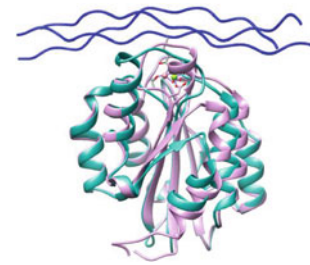


Fig. 11.2 Model of ligand binding and activation for I-domain integrins. The crystal structure of α_2 in its unbound form is represented in pink, PDB 1AOX [36]. This is overlaid onto the bound form of α2 which is represented in cyan PDB 1DZI [37]. Movements of the helices are highlighted with the arrows

11.6.1 Inhibitors

Inhibitors for I-domain containing integrins fall into two categories, competitive antagonists, and allosteric or non-competitive antagonists. Competitive antagonists bind in the region of the MIDAS domain on the α-chain. Only a few competitive inhibitors of I-domains have been identified. One such example is the AQC2 antibody which binds to the a1b1 I-domain [22, 61], competitively inhibiting collagen binding while maintaining the closed conformation of the I-domain. The snake venom of *Echis multisquamatus* (EMS16) binds competitively to the ligand-binding site of the α2 I-domain also maintaining the closed conformation of the MIDAS domain. Although it is a competitive antagonist, it does not interact directly with the MIDAS domain itself, however stearically blocks ligand binding [55]. The antibody AL-57 binds specifically to the activated/open αL MIDAS [117]. These inhibitors demonstrate that it is possible to develop competitive activation specific inhibitors for the I-domain integrins.

A group of small molecule αL ligand mimetics were designed based on the stucture of ICAM-1 as competitive antagonists to the MIDAS site [45, 62]. There is however some evidence to suggest that they bind to the ligand-binding site between the I-like domain MIDAS and the β-propeller (Fig. 11.3), also known as the linker site, or the α/β I-domain allosteric site [109, 119, 144, 151].

11.6.2 Allosteric Inhibition

The interaction of αLβ2 with ICAM provides a model for true allosteric inhibition of I-domain integrins. The carboxyl group of Glu^{34} on ICAM-1 co-ordinates directly with the Mg^{2+} in the MIDAS domain of αL [119]. Displacement of helices α1 and α7 occurs with ligand binding and provides the possibility of allosteric inhibition at the linker site. The I-domain of the αL subunit has been co-crystalized with a number of small molecule inhibitors [27, 32, 60, 77, 101, 140, 143] identifying a major site for allosteric inhibition of the I-domain (Fig. 11.4). Small molecules bind to a pocket in contact with the α7 helix on the opposite side of the molecule from the MIDAS domain [27, 32, 60, 101, 140, 143]. This region has been shown to be important for ligand binding and receptor function [20, 82]. Binding of small molecules do not cause any significant changes to the I-domain, suggesting that they do not activate the receptor. Kallen et al. suggest

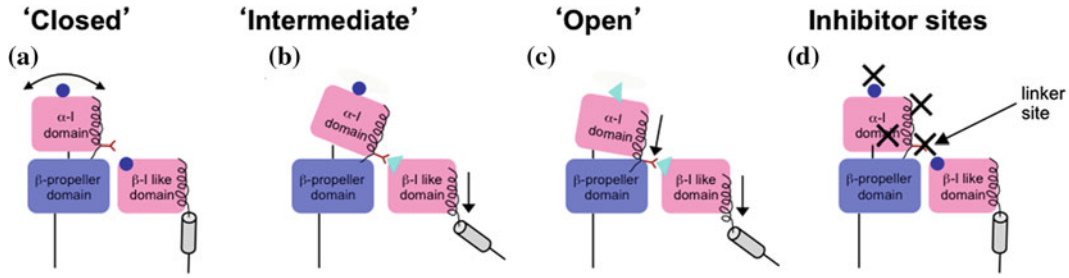


Fig. 11.3 I-domain integrin activation states and inhibitor sites. Figures a to c represent integrin activation states based on the model proposed for the complement receptor 4 [149]. The closed MIDAS site is represented as a blue circle. The open high affinity MIDAS site is represented as a cyan triangle.The α-I domain is thought to have a great deal of freedom in the closed state (**a**). Activation of the *β*-I domain MIDAS site and interaction between the MIDAS and the acidic residue (at the '*linker site*') locks the α-I domain at an angle (**b**). The α_7 helix moves downwards due to the activation of the α-I MIDAS site and reduces the angle of the α-I domain to the *β*-I domain (**c**). The sites that have been used to develop inhibitors are presented as black crosses in figure (**d**). This figure is modified from Cox et al. [25]

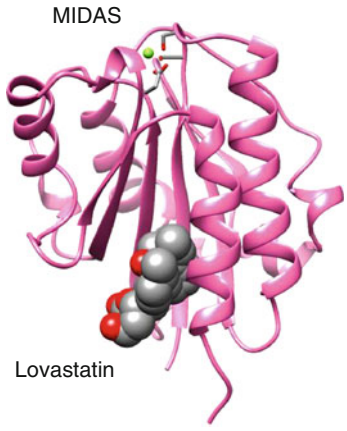


Fig. 11.4 Allosteric binding site on αL. Lovastatin is depicted in space fill in contact with the α7 helix. The binding site is on the opposite side of the molecule to the MIDAS, PDB 1CQP [60]

that they may inhibit signalling by locking the molecule in an inactive, low-affinity state by stabilizing the C-terminal α7 helix. Lovastatin and isoflurane have been co-crystallized in this position [60, 155]. Recently propofol has also been demonstrated to bind to this site [153]. These small molecules are proof of concept for the development of allosteric inhibitors for I-domain integrins that have no agonist activity.

Efalizumab binds to another region of αL which is also distal from the MIDAS [77]. It is largely in contact with the α1 helix, however its binding site is close enough to the MIDAS to cause stearic hindrence of ICAM-1 binding. Therefore it is possible that it acts as an allosteric inhibitor, however it is also possible that it is acting by sterically inhibiting ICAM-1 binding. The structure of efalizumab-bound αL adopts the unliganded, resting conformation. Efalizumab interacts with the α1 and α3 helices, and is thus thought to also act by stabilizing the closed, low affinity conformation. Therefore, there is structural evidence that competitive and non-competitive allosteric inhibitors can be developed for I-domain integrins.

11.7 Therapeutically Targeting I-Domain Integrins

The original anti-integrin drug discovery strategy was to develop monoclonal antibodies to the receptors and this has been commercially successful. A second strategy was to develop peptide antagonists usually based on the peptide sequence from the natural ligand (e.g., RGD) or snake venom peptides. Finally small molecule non-peptide antagonists have also been developed. As our understanding of the nature of the drug-integrin interaction has grown it has created the opportunity for developing different types of integrin antagonists.

11.7.1 Monoclonal Antibodies

Monoclonal antibodies provide an effective source of anti-integrins and were the initial strategy used. There are two types of antibody possible: complex-specific antibodies and subunit-specific antibodies. The advantage of complex-specific antibodies is that they are very specific and this can reduce the level of adverse effects. The specificity of subunit-specific antibodies depends on the target subunit. While the less specific antibodies may have increased adverse effects they may also be more effective as they target a complete family of receptors. The first anti-integrin antibody to be commercialized was the anti-β3 abciximab (ReoPro®). It is a potent inhibitor of platelet aggregation and has been extensively tested in clinical studies resulting in approval for use during percutaneous coronary intervention (PCI) [132, 133]. Natalizumab (Tysabri®) is an anti-α4β1/α4β7 antibody approved for multiple sclerosis [54, 99, 107] and has been shown to be beneficial in the treatment of Crohn's disease [131]. Efalizumab (Raptiva®) is an anti-αLβ2 antibody approved for the treatment of plaque psoriasis [48, 70, 95] and proved to be effective and safe for long-term use [74] although it has recently been withdrawn from the market.

11.7.2 Peptide-Based Inhibitors

Peptide-based antagonists of integrins have been very attractive for a number of reasons primarily because short peptide sequences that mediate integrin binding were identified from integrin-binding proteins, e.g. RGD, and the effectiveness of phage display libraries at identifying novel integrin-binding sequences. In most cases cyclic peptides are used due to their enhanced stability and potency. Snake venoms are a rich source of bioactive molecules. Snake C-type lectins (snaclecs) have been found to modulate haemostasis. Rhinocetin is a snaclec isolated from the venom of *Bitis gabonica rhinoceros* and is an α2β1 antagonist. It blocks collagen-induced platelet activation [137]. Rhodocetin from *Calloselasma rhodostoma* [13, 35], EMS16 from *Echis multisquamatus* [80] and VP12 from *Vipera palestina* [124] are all snaclecs that inhibit α2β1. Del-1 is a 52 kDa natural inhibitor of αLβ2 and administration of it has anti-inflammatory effects in diseases such as periodontitis [38].

11.7.3 Non-peptide Small Molecule Antagonists

The ideal drug is an orally active non-peptide small molecule and this has been the goal in anti-integrin therapy. While there are many small molecule inhibitors in pre-clinical development tirofiban (Aggrastat®) is still the only approved non-peptide inhibitor. It is an αIIbβ3 antagonist although it has no oral activity and like eptifibatide [57] its development was based on a viper venom peptide (echistatin). It was approved for use in PCI and acute coronary syndromes [134]. αLβ2 has attracted a lot of interest with the discovery of non-peptide inhibitors with nM IC_{50} values [32, 45, 46, 63, 78, 101, 102, 140, 141, 147]. BMS-587101 a small molecule αLβ2 antagonist reduces symptoms in a mouse model of RA [128]. A few of the compounds showed efficacy in mouse models of inflammation but it is still unclear what their target disease is likely to be. SAR 1118 [45] blocks αLβ2 and is undergoing clinical trials in diabetic macular oedema [97] and dry eye [114]. αMβ2 small molecule agonists enhance cell adhesion and thus reduce chemotaxis [79]. See Fig. 11.5 for sample structures.

11.7.4 Collagen and Cadherin Receptor Antagonists

α1β1 is a collagen and laminin receptor and has been implicated in angiogenesis and fibrosis and diabetic neuropathy. Jerdostatin [112], viperistatin [67], lebestatin [94] and obtustatin [88] are snake venom disintegrins, which have low micromolar potency and high selectivity against α1β1. Their selectivity is due to the presence of

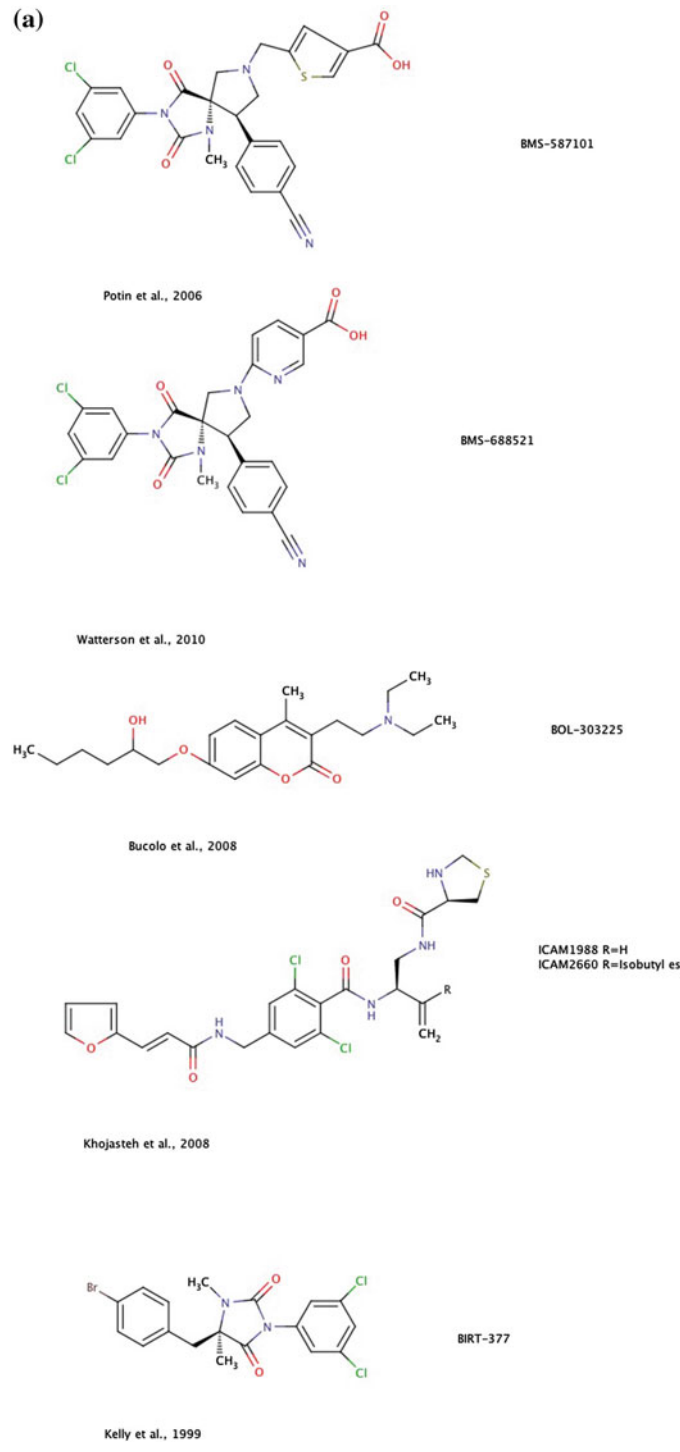
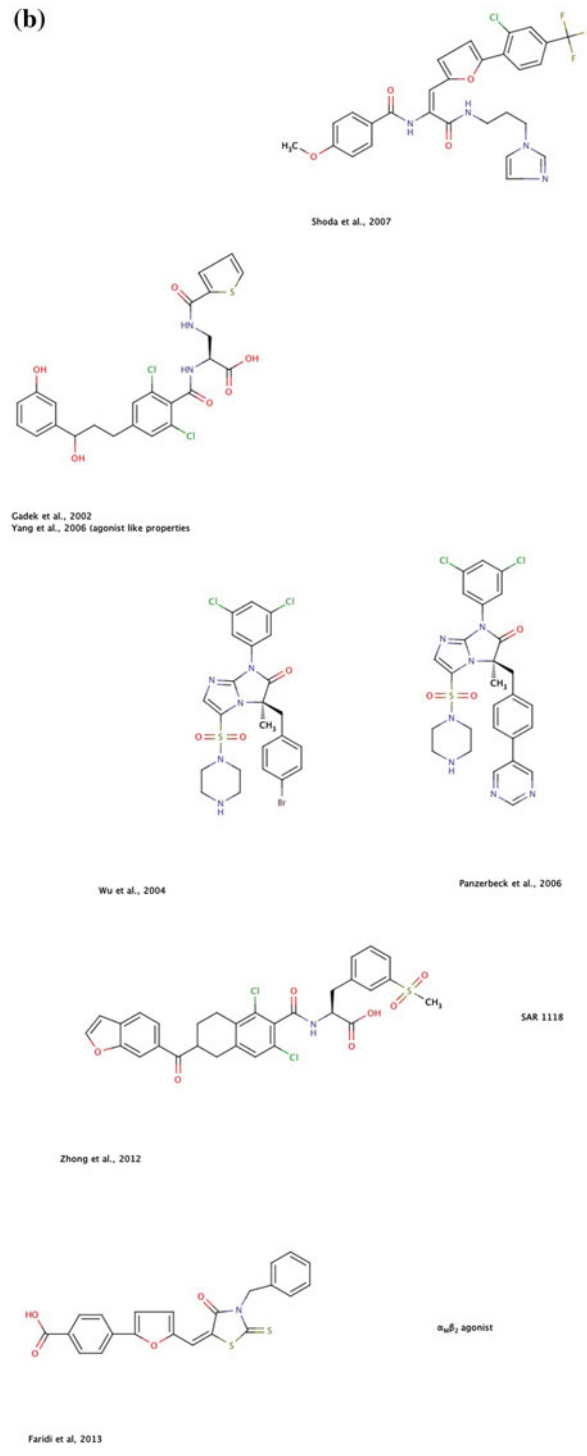


Fig. 11.5 Some structures of small molecule $\alpha L\beta 2$ antagonists. Sample structures of small molecule $\alpha L\beta 2$ antagonists

Fig. 11.5 continued



the KTS sequence and the flanking residues further enhance their potency. Thus, viperistatin, which contains KTSR, is more potent than obtustatin, which contains KTSL [15, 67]. Jerdiostatin contains the sequence RTS rather than KTS [112]. These have been shown to be effective in models of angiogenesis [81] and melanoma metastasis [18, 124]. However, there is no evidence of small molecule $\alpha 1\beta 1$ antagonists in development probably due to a lack of a clear target disease for an antagonist.

$\alpha 2\beta 1$ is also a collagen and laminin receptor but its presence on platelets where it is an important collagen receptor mediating thrombus formation has made it an attractive target for drug discovery. As with many integrins there are snake venom C-type lectin related proteins that specifically target it. These include rhinocetin [137] rhodocetin [37], vixapatin (VP12) [124], EMS16 [80], flavocetin [4] and VP-I [5]. Potent small molecule inhibitors have also been discovered with sub-micromolar IC_{50} values [84, 92, 93] (see Fig. 11.6) for sample structures. These compounds have been tested in a number of different disease models. Vixapatin was shown to be effective in a model of angiogenesis [86, 110], melanoma metastasis [124] and thrombosis [93]

$\alpha 10\beta 1$ and $\alpha 11\beta 1$ are collagen receptors and αE is an E-cadherin receptor. However, these are newly discovered receptors and there is no evidence of any antagonists under development.

11.7.5 β2 Antagonists

$\alpha L\beta 2$ is an ICAM receptor and is an active target for drug discovery. One of the first $\alpha L\beta 2$ antagonists was the humanized monoclonal antibody efalizumab [29, 49]. This was investigated for use in psoriasis [48, 75] and was ultimately approved for this indication. It was also investigated for use in renal transplant [138]. While it was shown to be effective in plaque psoriasis [74] it was ultimately withdrawn from the market due to an increase in the incidence of progressive multifocal leukoencephalopathy (PML) [69]. PML is a serious disease due to reactivation of a JC virus infection in the brain. The original infection is usually asymptomatic but re-activation of the infection leads to demyelination and is associated with a high level of mortality. Once primarily associated with HIV it has more recently been associated with biological immunosuppressive therapy especially natalizumab, efalizumab and rituximab [130]. While the response of Élan/Biogen Idec to the occurrence of PML with natalizumab was to develop a Risk Evaluation and Mitigation Strategy (REMS) in conjunction with the FDA along with a black-box warning, Genentech opted to withdraw efalizumab from the market.

Other antibodies to $\beta 2$ integrins have been described. Hu23F2G [42] and rhuMAb CD18 [7] are anti-$\beta 2$ antibodies that failed to reduce infarct size after angioplasty or thrombolysis respectively. There were also small phase II studies with rhuMAb CD18 in haemorrhagic stroke [105] and Hu23F2G in multiple sclerosis [12]. AL-57 is a monoclonal antibody that specifically recognizes activated $\alpha L\beta 2$ [117]. UK-279,276 is a recombinant glycoprotein also known as Neutrophil Inhibitory Factor [136] that selectively binds to $\alpha M\beta 2$ [72] and investigated for use in stroke although its clinical development appears to be ended. Phage display has been used to identify a peptide-based inhibitor of $\alpha M\beta 2$ [56].

BMS-587101 is a small molecule $\alpha L\beta 2$ antagonist that entered clinical trials for transplant rejection [101]. It also showed benefit in an animal model of rheumatoid arthritis [128]. BMS-688521 is a more potent follow-on compound [141]. BOL-303225-A is a coumarin derivative that has inhibitory activity against both $\alpha L\beta 2$ and $\alpha M\beta 2$ [17]. ICAM1988 is the active metabolite of the small molecule prodrug ICAM2660 that inhibits $\alpha L\beta 2$ [65]. Virtual screening has also identified potential small molecule $\alpha L\beta 2$ antagonists [120]. A number of other groups have also discovered small molecule $\alpha L\beta 2$ antagonists [45, 62, 63, 96, 98, 147] (see Fig. 11.5).

Lifitegrast (SAR 1118) is an $\alpha L\beta 2$ antagonist [156] that is undergoing clinical development in the area of ocular inflammation. It has undergone both phase I [97] and phase II [114] trials. It is

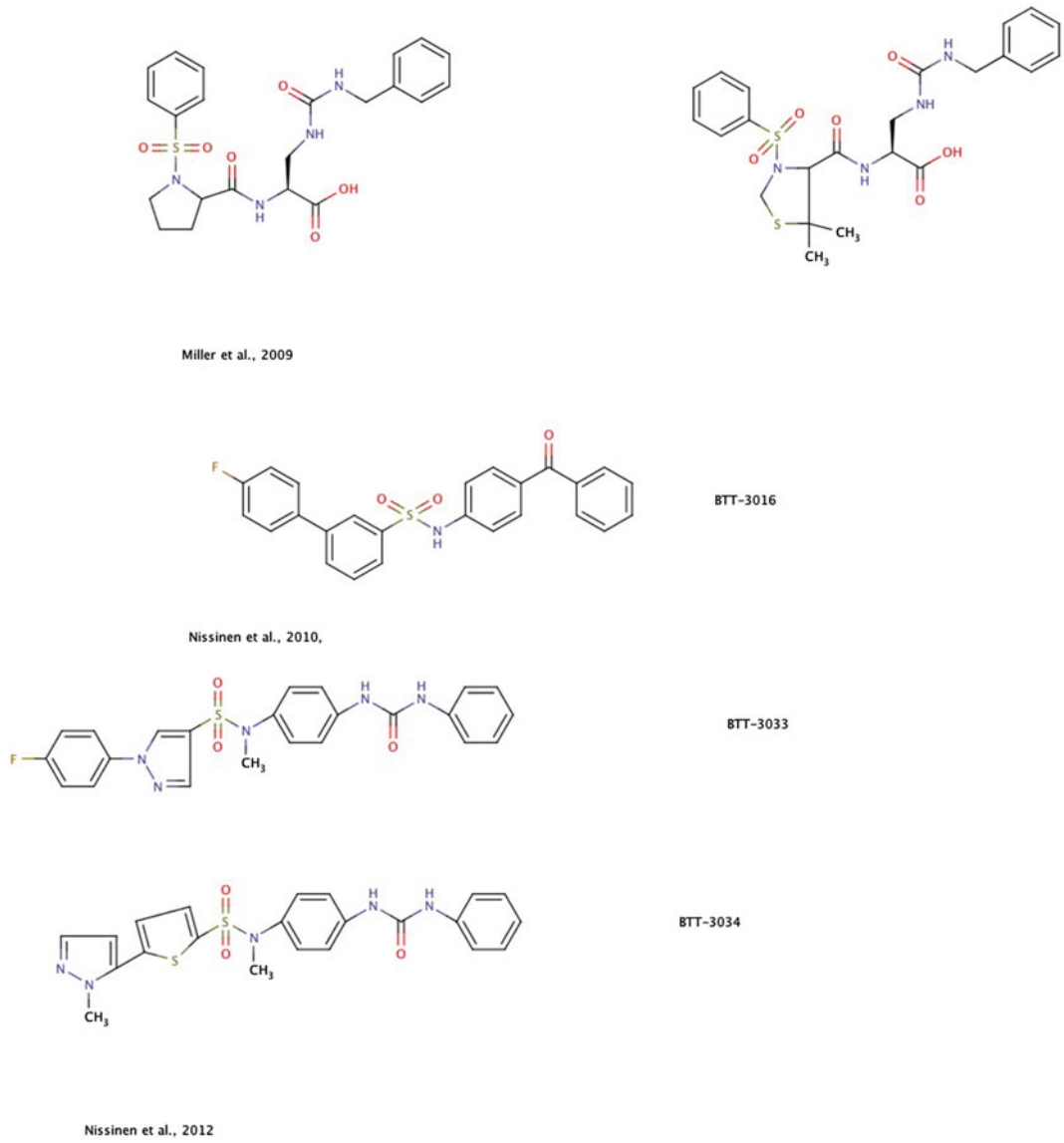


Fig. 11.6 Some structures of small molecule $\alpha 2\beta 1$ antagonists. Sample structures of small molecule $\alpha 2\beta 1$ antagonists

being investigated for dry eye [114] and diabetic macular oedema [97]. Currently this is likely to be the first I-domain antagonist to be commercialised. $\alpha L\beta 2$ antagonists have also shown potential benefit in lupus [135], renal transplant [138], psoriasis [34, 75] and experimental autoimmune encephalomyelitis [139]. However, there has been a very high failure rate in the clinical development programmes for $\beta 2$ integrin antagonists [33, 53], although it is worth noting that the failed trials tended to focus on cardiovascular indications such as reperfusion injury, myocardial infarction, and stroke and thus may only reflect these indications. The $\beta 2$ integrin chain binds LPS and the region between amino acids 266–318 in the A domain has been identified as the LPS binding site [146]. This peptide has been shown to be effective in a mouse model of sepsis [145].

The $\alpha IIb\beta 3$ discovery programme was severely impacted by the nature of the interaction

between the drugs and the receptor [24, 25]. Rather than being pure antagonists many of the inhibitors had agonist activity [26]. This is also true of αLβ2 as compounds that were previously identified as allosteric inhibitors have been shown to be αLβ2 agonists [150] and a number of compounds have been found to have some agonist like properties similar to that seen with αIIbβ3 antagonists [119]. Pure agonists have also been discovered [79] and are known as leukadherins [40]. The explanation for this lies in the concept of permissive antagonism [64]. The conventional view of integrin function is that the agonist binds to the receptor, induces conformational changes leading to receptor clustering and finally to outside-in signalling and that this happens in a linear fashion. Leukadherins appear to bind to the receptor at an allosteric site that facilitates ligand binding. However, the leukadherins also block receptor clustering and outside-in signalling and thus are antagonists. So leukadherins are best described as permissive antagonists.

11.7.6 Problems with Integrin Antagonists

As many integrin antagonists are designed around the natural ligand for the receptors such as RGD it is not surprising that the resulting antagonists often display agonist-like activity [142]. RGD-based αVβ3 and αVβ5 inhibitors were found to stimulate angiogenesis at low doses [104]. This has also been seen with oral αIIbβ3 antagonists where low doses were shown to induce platelet aggregation while higher doses were inhibitory [26]. In both cases the problems appear to arise during trough periods. In the case of αIIbβ3 antagonists this is not a problem with the intravenous agents, as these are maintained at high plasma concentrations using an infusion. However, it was a bigger problem for the oral compounds. A similar situation exists with the I-domain integrin antagonists where compounds have significant agonist-like activity [40, 79, 150]. It is not yet clear whether this will prove to be a problem for the development programme. Another problem identified with I-domain integrin antagonists is PML, which led to the withdrawal of efalizumab from the market [69]. It is not clear whether this is unique to efalizumab or is only associated with the use of biological agents or could happen with any antagonist of αLβ2. Clearly this will be an issue that will have to be addressed in the development programme for any αLβ2 antagonist and if it is an issue companies will need to decide whether they will stop the development cycle or implement a REMS.

11.7.7 Modulation of Integrin Expression

In many cases integrin expression on the cell surface is dynamic and is regulated to modulate cell function. This is important in processes such as tumour metastasis where tumour cells must loose their adhesive properties to metastasise and must gain new adhesive properties to colonise the target organ. The expression of some integrins especially the β2-integrins is controlled by micro RNAs and this creates the potential for using specific micro RNA to influence the expression of individual integrins [21]. The tumour suppressor genes tuberous sclerosis complex (TSC) regulates α1β1 expression and thus cell migration [85].

11.8 Conclusions

Integrins were discovered almost 30-years ago at a time when the pharmaceutical industry was undergoing a paradigm-shift from chemistry-led drug discovery to target-led drug discovery. Prior to this, drug discovery projects typically involved screening a library of compounds for activity in a disease model. The discovery of integrins and their recognition sequence RGD allowed for a different approach to drug discovery where pharmacologists screen chemical libraries for activity on a specific receptor. The big advantage of a chemistry-led approach is that it produces a drug with desirable activity even if its mechanism of action is unknown. On the other hand a target-led approach

makes high-throughput screening a possibility and allows for the discovery of drugs with a known mechanism of action. However, the success of a target-led approach is dependent on the biology of the target being well understood, i.e., a validated target, integrins have proven to be a difficult drug target to commercialize, despite their importance in thrombosis, autoimmune disease and cancer only 4 agents are on the market and three of these are in the field of thrombosis.

There are a few key factors necessary for a successful drug discovery programme. These include a validated target for a specific disease, highly potent and specific ligands, a good ADME (Absorption, Distribution, Metabolism, Excretion) profile and low toxicity. As integrins are large receptors binding to equally large ligands the possibility of developing small molecule antagonists would be expected to be the major challenge; however small molecule antagonists have been developed for many of the integrins. This was facilitated by the identification of short binding motifs such as RGD. Another obvious problem is the development of specific inhibitors, which is a challenge as many of the integrins bind the same motifs such as RGD. Yet highly specific small molecule inhibitors were developed for many of the integrins.

A poor ADME profile is one of the major reasons for a drug failing in the development pipeline. The use of monoclonal antibodies overcomes many of the ADME problems with the exception of absorption. However, they are often dosed on a monthly basis, which mitigates the lack of oral activity. Monoclonal antibodies were approved for all of the successfully targeted integrins (αIIbβ3, α4β1 and αLβ2). Small molecule antagonists were also approved for αIIbβ3 although they were not orally active. Orally active antagonists were also investigated but these all failed in part due to poor ADME profile.

Toxicity is another major reason for failure of drugs in development. This has been a problem for many integrin antagonists. All of the orally active αIIbβ3 antagonists failed due to increased cardiovascular mortality. This was in part due to a poor ADME profile but it was also due to the presence of significant agonist-like activity with the drugs. Both natalizumab (anti-α4 antibody) and efalizumab (anti-αLβ2 antibody) were associated with PML, a very serious adverse effect that ultimately led to the withdrawal of efalizumab from the market. However, this is due to their immune suppressive effects, which is a function of the targets themselves.

The lack of validated targets appears to be the major hurdle in developing anti-integrin antagonists. It is not surprising that the most successful integrin target was αIIbβ3 as it is specific for platelets and was found to play a unique and critical role in platelet function. It was also clear that those patients who lacked αIIbβ3 (Glanzmann's thrombasthenia) had a complete lack of platelet function. Yet despite the presence of a platelet-specific, validated target αIIbβ3 antagonists did not live up to the expectation that they would be the next generation "super-aspirin" and instead are restricted to high-risk patients. It was the $P2Y_{12}$ ADP receptor antagonists such as clopidogrel and prasugrel, which were to become the anti-platelet agent of choice and ultimately become one of the biggest selling drugs today. While α4 integrins were known to play a role in lymphocyte function they were only one of many integrins found on lymphocytes so it was by no means certain that the anti-α4 antibody natalizumab would be successful in multiple sclerosis and Crohn's disease.

There is a lack of validated targets for I-domain integrins. The most obvious target disease for the collagen receptors is thrombosis where collagen-induced platelet activation is important and Biotie have been developing small molecule $\alpha2\beta1$ antagonists for thrombosis. However, the success of αIIbβ3 antagonists and $P2Y_{12}$ receptor antagonists such as clopidogrel and prasugrel suggest that there is no market for a platelet collagen receptor antagonist even if it was effective or at least it will be difficult to convince a pharmaceutical company to enter this field. Currently the $\alpha2\beta1$ project is not listed as an active project with Biotie. Collagen receptors, like many other integrins, also suffer from the problem of redundancy as many cell types (including platelets) contain multiple collagen receptors suggesting that blocking any one

specific receptor may not produce a strong pharmacological effect. Furthermore the widespread distribution of collagen receptors also suggests a potential for adverse effects. There is interest in $\alpha 10\beta 1$ as a chondrocyte biomarker and important in cartilage production although it is not clear if there is a role for drugs that target $\alpha 10\beta 1$. Fibrosis is an important therapeutic area where collagen-binding integrins are very important [57]. However, there are also other integrins involved such as αV integrins and thus the integrins that should be specifically targeted has yet to be elucidated.

$\alpha L\beta 2$ is probably the most investigated I-domain integrin however; the lack of a validated target has proven a problem. Initial targets focused on reperfusion injury post-MI but this did not produce significant clinical results. Typical of the strategy used by pharmaceutical companies they all pursued the same target with the same result. Ultimately the problem was not a lack of potent inhibitors but a lack of understanding of reperfusion injury. However, when a drug fails in a clinical study history has shown that a company is more likely to drop the drug and target entirely rather than investigating other potential uses of the drug.

So what is the future for integrin antagonists and more specifically I-domain antagonists? $\beta 2$ integrins are important in the immune response but α4 integrin antagonists are the first to market in this space. It will be important to identify a disease in which $\beta 2$ integrins are more significant than α4 integrins. Certainly efalizumab was effective in psoriasis as it was clinically approved and also showed benefit in renal transplant. Ocular inflammation appears to be the most advanced therapeutic area for $\alpha L\beta 2$ inhibitors. Ultimately the problem of toxicity, especially PML will need to be addressed. Was this unique to efalizumab or will it be a problem for all $\beta 2$ antagonists? At least in ocular inflammation this is unlikely to be an issue as there is low systemic exposure to the drug. Cancer is another important area for anti-integrins and there has been a lot of interest in this for decades however, an anti-integrin has yet to be approved in this area. Currently the major focus is on $\alpha V\beta 3$ inhibitors but I-domain collagen-binding integrins appear to play a significant role in angiogenesis, which may make them potential targets in cancer. Infection is another potential target disease as a number of I-domain integrins are involved in infection of cells by bacteria and viruses. This is the new area of targeting host factors such as adhesion receptors to supplement anti-microbial therapy.

Ultimately the future of anti-integrin pharmacology lies in further work on the role of integrins in disease. Advances in our understanding of integrin structure means that we can now develop better antagonists that are activation-specific and that are not partial agonists. This needs to be supplemented by a better understanding of the role of integrins in health and disease if successful therapeutics are to be developed.

11.9 Perspectives

Potent antibodies and small molecule inhibitors for I-domain integrins have been discovered and a number of the antibodies have entered into clinical development programmes. However, none have proven to be successful either due to lack of efficacy or adverse effects. Since I-domain integrins are known to be important in cancer, autoimmune disease and fibrosis, all areas where there are significant unmet needs, there will remain interest in these receptors as potential drug targets. A key issue to be addressed with I-domain integrins is identification of validated targets. As multiple integrins are often involved in these target diseases further research on specific integrins and their roles in the disease process will be required to ensure that the appropriate integrin is being targeted in each disease. This in conjunction with recent studies that elucidated the molecular interaction between antagonists and their target integrins will allow new generations of potent and specific antagonists to be tested in theses diseases.

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