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FIBRONECTIN-INTEGRIN INTERACTIONS

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1. ABSTRACT

Fibronectin is recognized by at least ten cell surface receptors of the integrin family. Most cell types in the body can adhere to fibronectin via these receptors, and thereby fibronectin becomes involved in many different biological processes. Three areas related to fibronectin and its receptors which have developed rapidly during the last few years are summarized in this review: the mechanisms of interactions between fibronectin and integrins, fibronectin polymerization, and *in vivo* functions of the proteins as studied by gene targeting in mice.

2. INTRODUCTION

The interaction of cells with adhesion proteins in the extracellular matrix (ECM) provides signals which affect the morphology, motility, gene expression and survival of adherent cells (1-3). Many cell adhesion proteins have been identified and extensively characterized. In general, they are large, multifunctional mosaic proteins; some of them can be grouped into protein families, but otherwise they are structurally very diverse. Since each cell adhesion protein has specific effects on cells, their temporal and spatial deposition in ECMs is important for various normal and pathological processes such as formation, maintenance and remodeling of tissues, lymphocyte recirculation, thrombus formation , tumor formation and metastasis.

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In contrast to the ligands, most cellular receptors for adhesion proteins of the ECM belong to recirculation, thrombus formation, tumor formation and metastasis.

One protein family, the integrins (1). Additional adhesion receptors for the ECM clearly exist, *e.g.* dystroglycan (4, 5) and syndecans (6, 7), but the integrins have a dominating role in the anchoring of cells to the ECM. At present, 22 different integrins and several additional splice variants have been identified, a specific subset of them being expressed by each cell. Thus, the appropriate capacity for receiving information from ECMs of varying composition is available for the cells.

The topic of this review is the interaction of one cell adhesion protein, fibronectin, with its various integrin receptors. The focus is on the mechanisms of ligand binding, and the importance of the interactions for fibronectin fibril formation. In addition, the *in vivo* effects in mouse of disruption of the genes for fibronectin and integrins subunits are discussed. For information about the intracellular responses following fibronectin-integrin interactions, other recent reviews are recommended (8-10).

3. DISCUSSION

3.1. Fibronectin

Fibronectin is a prototype cell adhesion protein, widely distributed in the tissues of all vertebrates and a potential ligand for most cell types (11). It is present as a polymeric fibrillar network in the ECM and as soluble protomers in body fluids. The protomer consists of two subunits linked in an antiparallel manner by a pair of disulfide bridges at

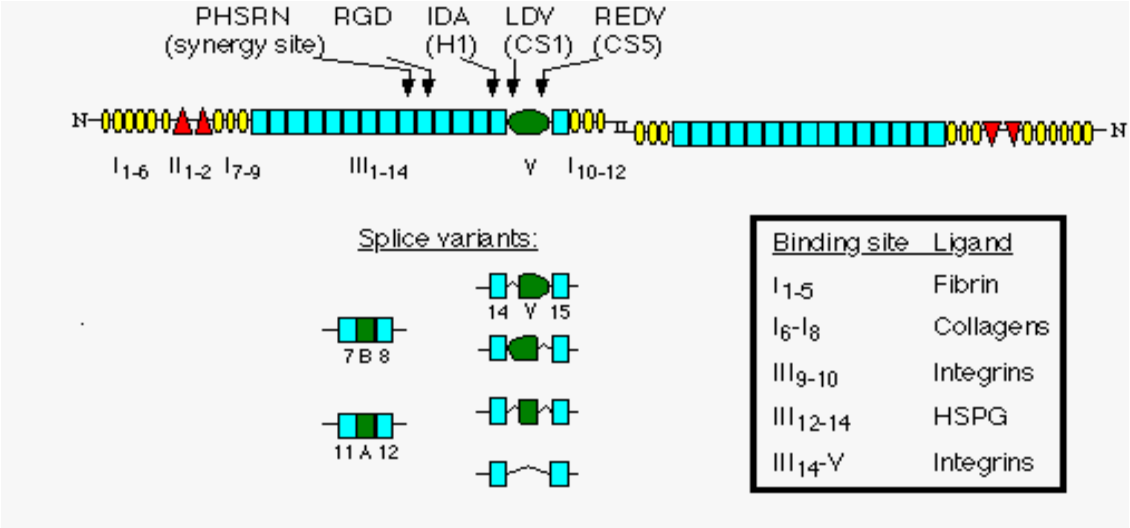

Figure 1. Schematic model of a plasma fibronectin protomer. The protein is a dimer of two subunits which are identical except for the inclusion of the V-segment in one of the chains. The subunits are covalently connected close to the C-termini by two disulfide bridges. Three types of homologous structural units called I, II, and III make up most of the protein (represented by small ovals, triangles, and squares, respectively). Integrin binding sites are indicated in one of the subunits by arrows. The fibronectin found in blood plasma is a major form of the protein, but other splice variants are present at other locations and at specific developmental stages. The alternatively spliced exons (III_A, III_B, and V) are marked in green and may be present in one or both of the subunits. The known variants of the splicing reactions are illustrated.

the C-termini. Fibronectin is a good example of a mosaic protein since essentially the entire primary sequence, except for the V segment, is comprised of three types of repeating amino acid motifs (Fig. 1). Several variants of the protein are formed by alternative splicing of the pre-mRNA at three positions: the type III repeat units A and B can independently be included or excluded, while the V segment is subject for a more complex splicing pattern (Fig. 1).

Several of the repeating units function together as distinct domains with specific biological activities (12). The domains are separated by short hinge regions which allow the molecule to adopt different overall shapes. The native soluble protomer is believed to have a globular configuration (13, 14), while an elongated form would be adopted during polymerization to fibrils (15, 16). However, it should be noted that the procedures for isolation of fibronectin require unphysiological conditions (4 M urea is commonly used), hence the native state of the protein is really not known. Conformational changes in the fibronectin molecule have been demonstrated to be key events for some of its functions (17-20), but it is not known if the regulation of function is related to the gross shape alterations or to more subtle structural rearrangements.

Two regions in each fibronectin subunit possess cell binding activity: III_{9-10} and III_{14}-V. The amino acid sequence RGD, a widely occurring cell adhesive motif originally discovered in fibronectin (21), is located in III_{10} The RGD motif in fibronectin and other cell adhesion proteins is the most important recognition site for about half of all known integrins. The affinity for short peptides containing the RGD sequence varies significantly among these integrins (22, 23), but for all receptors the affinity for larger protein fragments or the intact proteins is orders of magnitude higher than for short RGD-containing peptides(24, 25). This indicates that areas in the vicinity of the RGD site contribute to the contact surface between ligands and integrins. In fibronectin, one such site of major importance for the interaction with some of the fibronectin binding integrins has been located to III_9 (26) This site, which acts in synergy with the RGD site, has the minimal amino acid sequence PHSRN(27) (Table 1, Figs. 1 and 2).

The III_{14}-V region is recognized by two integrins (Table 1), and for both of these receptors three binding sites have been identified within this region (Fig. 1). The so called CS1 site has approximately 20-fold higher affinity for the integrins than the other two sites, namely CS5 and H1 (32). However, CS1 as well as CS5 can be spliced out and are therefore available only in some fibronectin molecules, while H1 is present in all forms of the protein. The related sequence motifs LDV and IDA are the key cell binding structures in CS1 and H1, respectively (33, 34). The active site in CS5 has been identified as REDV in human fibronectin, corresponding to RGDV in the rat and

Table 1. Fibronectin-binding integrins.

Receptor	Main binding site in FN[2]	Additional binding sites in FN	Other protein ligands
α3β1	RGD		LN 5
α4β1	LDV in CS1	H1, CS5, (RGD)	VCAM-1
α5β1	RGD	PHSRN	
α8β1	RGD		VN, TN
αVβ1	RGD		
αVβ3	RGD		VN, OP, vWF, FG, TSP
αIIbβ3[1]	RGD	PHSRN	FG, vWF, VN
αVβ6	RGD		TN
α4β7	LDV in CS1		MAdCAM-1, VCAM-1
α?β8	CS1		

[1]Unactivated αIIbβ3 in resting platelets does not bind soluble ligands except short RGD peptides, but it can mediate adhesion to surfaces of immobilized fibrinogen or RGD peptide (28). The activated receptor can bind all indicated ligands in soluble as well as immobilized form (29, 30).
[2]Abbreviations: FN, fibronectin; FG, fibrinogen; LN, laminin; MAdCAM, mucosal addressin cell adhesion molecule; OP, osteopontin; TN, tenascin; TSP, thrombospondin; VCAM, vascular cell adhesion molecule; VN, vitronectin; vWF, von Willebrand factor.

bovine proteins (35). Thus, the binding sequence in CS5 shows similarity to both the RGD and the LDV motifs. It is worth noting that integrin binding sites in fibronectin as well as in other proteins usually contain an aspartic acid as one of the critical amino acids.

3.2. Integrins

Most integrins function in cell-matrix contacts, some mediate cell-cell interactions, and a few participate in both types of contacts. (1) All integrins are composed of two noncovalently associated subunits, denoted α and β, which both span the plasma membrane. The combination of α and β subunits determine the specificity for extracellular ligands as well as intracellular signaling events.

A striking feature of many integrins is the ability to bind multiple ligands. For example, integrin αVβ3 serves as a receptor for vitronectin, fibronectin, fibrinogen, von Willenbrand factor, thrombospondin, and osteopontin (36). Since each of these proteins contain an RGD sequence as the dominating recognition motif for integrin αVβ3, the ligands can be envisaged to bind to one common binding site in the receptor. In the case of integrin α1β1, laminins and collagens are recognized as ligands, although the two groups of proteins have no known structural similarities. In spite of this and several other examples, there are no indications that there would be separate binding sites for different ligands on an integrin (although the possibility remains open).

It is also commonly found that a particular cell adhesion protein can bind to more than one type of integrin. Fibronectin is an extreme case, for which binding to ten different integrins have been described (Table 1). This should not be interpreted as redundancy of receptors which could compensate for each other if necessary; rather, it is clear that different intracellular signals are generated by the ligand depending on which integrin it interacts with. The point is most convincingly illustrated by the strikingly different mouse phenotypes obtained after disruption of the various integrin genes (see below) (37, 38).

3.3. Molecular mechanisms of ligand-integrin interactions

A central concept for the function of integrins is their ability to shift between active and inactive ligand binding states, by alteration of the conformation of the extracellular domain (39). Transitions between the two states are dynamically regulated by the cell through energy dependent events involving the cytoplasmic tails of the integrin (40). A third conformational state is acquired after ligand-occupation, as demonstrated by exposure of epitopes called ligand-induced binding sites (LIBS) for antibodies. Experimentally, the three conformations can be induced or stabilized by the binding of different monoclonal antibodies to the α or β subunits at epitopes distinct from the ligand binding site (39). In adherent cells the integrins are mainly in the active state, but possibly become inactivated during specific situations, *e.g.* cell migration and mitosis. In circulating cells the integrins are present on the cell surface in an inactive conformation until the cells are exposed to factors which trigger intracellular reactions leading to activation of the integrins (41, 42).

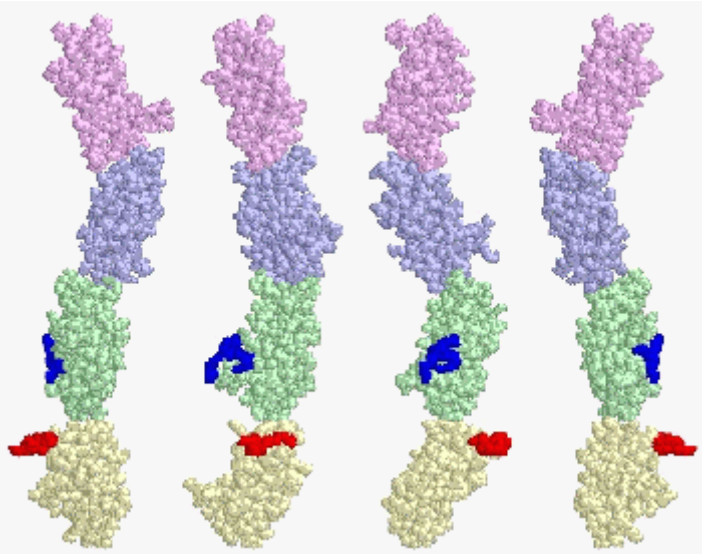

Figure 2. Structure of the fibronectin fragment III_{7-10} as determined by X-ray crystallography of the recombinant protein fragment. The fragment is presented in four different angles with a stepwise rotation of 60 degrees. The four repeats are shown in different colors: pale purple, blue, green, and yellow for III_7-III_{10}, respectively. The synergy site (PHSRN) in III_9 repeat is marked in blue color, and the G**RGD**S loop in III_{10} is marked in red. In fibronectin, as well as in many other proteins, several type III units link to each other as an extended array. The interfaces between the units in fragment III_{7-10} are highly variable, suggesting that they may be important for the structure and interactions of the protein. The interdomain surface between III_9 and III_{10} is smaller than between other units in FN_{7-10}. Therefore, they are likely to interact less strongly, and this junction may represent a flexible hinge region. Furthermore, the link between III_9 and III_{10} has an unusually small rotation, resulting in the exposure of the RGD loop in III_{10} and the synergy site in III_9 on the same side of the molecule. The two sites are separated by 30-40Å, a distance which one integrin molecule could span. The RGD loop is well exposed extending ~10Å away from the body of the molecule. These structure data were obtained from Leahy *et al.*, 1996 (31).

Intimately related to the conformational rearrangements in integrins is their requirements for extracellular divalent cations for ligand binding ability. There is evidence for at least three functionally important coordination sites for divalent cations in the extracellular integrin domains, each one exhibiting different ion preference (43). Mg^{2+} is most likely the dominating ion which supports ligand binding to integrins under normal physiological conditions. Mn^{2+} stimulates ligand binding to integrins even more strongly, but it is questionable whether this is of physiological relevance. The Mn^{2+} effect is due to the fact that the ion can support ligand binding, possibly by the same mechanism as Mg^{2+}. Additionally, Mn^{2+} is capable of inducing/stabilizing a ligand binding conformation of otherwise inactive receptors (43, 44). Thus, Mn^{2+} can activate integrins independently of the intracellular events which normally regulate the extracellular conformation of the receptor (*i.e.* in the presence of Mg^{2+}). In contrast, Ca^{2+} at millimolar concentrations has an inhibitory effect on the ligand binding ability of most integrins, by inducing the inactive conformation. However, the role of Ca^{2+} is complex since low concentrations (μM) appear to stimulate ligand binding. This indicates that Ca^{2+} can bind to at least two different sites in integrins (43-45). The number of binding sites for Mn^{2+} has been directly investigated in one study; by equilibrium gel filtration three Mn^{2+} ions were found to bind to each αIIbβ3 molecule (46). It is unknown whether Mg^{2+} binds to more than one site in integrins, and it is also unclear if any of the metal ion (Me^{2+}) sites can accept alternative divalent ions.

Several good candidates for ion coordination sites have been identified in fibronectin binding integrins; different α subunits contain three or four homologous sequences (DxDxDGxxD) which are similar to the Ca^{2+}-binding EF-hand motif (47), and a second type of cation binding motif, DxSxS, is located in the β subunits (Fig. 3) (48, 49).

Direct binding of divalent cations has been demonstrated for the latter site; a 14 amino acid peptide from the β3 subunit ($β3_{118-131}$; shown in Fig. 3), which is strongly conserved among all β subunits, was found to bind Mn^{2+}, Mg^{2+}, or Ca^{2+} with 1:1 stochiometry (46). It is not known if this site has an equally broad ion specificity in the intact receptor. Interestingly, $β3_{118-131}$ could also bind RGD peptides (46). It had previously been suggested that one of the integrin-bound ions may be directly involved in bridging to an aspartic acid residue of the ligand (*e.g* RGD or LDV sites) (50). However, the RGD

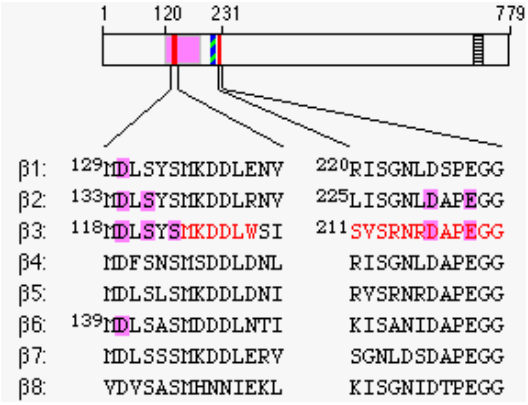

Figure 3. Schematic illustration of the integrin subunit β1. The numbers refer to amino acids starting from the N-terminus of the mature protein. The horizontal black stripes represent the transmembrane part. Both inhibiting and activating monoclonal antibodies bind to the small segment of the protein marked with alternating blue and green lines. The dotted pink and red boxes indicate regions corresponding to those which have been implicated in ligand binding in αIIbβ3; RGD peptides have been crosslinked mainly to the pink region, while direct binding of ligands have been demonstrated for the isolated red regions. The red regions are highly conserved among all known β subunits, as shown by the aligned amino acid sequences. Mutation of anyone of the oxygenated amino acids shaded in pink to alanine has been shown to result in inability of the receptor to bind ligand, presumably because coordination of a divalent cation by these amino acids is required for the active protein conformation.

peptides bound also to a mutant form (D119A) of $\beta3_{118\text{-}131}$ which lacks the ion binding ability. Furthermore, the ion bound to the normal $\beta3_{118\text{-}131}$ was displaced upon addition of RGD peptides. Ligand binding to an intact receptor had a similar effect, causing two of the three Mn^{2+} ions bound to αIIbβ3 to be released (46). Thus, the interactions between integrins and RGD-containing ligands appear to be independent of a direct involvement of divalent ions, but ions and ligands mutually influence their interactions with the receptor through allosteric regulation of the receptor conformation.

3.4. Ligand specificity of fibronectin binding integrins

Integrins α5β1, αIIbβ3, and α4β1 have been studied in much greater detail than the other fibronectin-binding integrins. These receptors are therefore discussed first, followed by the others in the same order as they appear in Table 1.

Integrin α5β1: This integrin is a prototype fibronectin receptor in the sense that it was the first to be identified (51), and, in contrast to most other fibronectin binding integrins, it is specialized for binding this ligand. α5β1 is expressed by many cell types and is probably the major fibronectin receptor in several of these.

The RGD loop in III_{10} is the critical recognition site for α5β1 (21), but the synergy site PHSRN in III_9 is required for high affinity binding (27). Screening of peptide libraries for binding to isolated α5β1 by the phage display technique enriched peptides containing the sequence RGDGW/F (52). Notably, the replacement of the R for a K abolished the binding to α5β1, while αIIbβ3 bound almost equally well to both variants (53).

A curious finding from the phage display screening was the strong and specific binding of the peptide RRETAWA to α5β1 (52). The peptide did not bind to αVβ3 and only weakly to αVβ1. There is no sequence homology between this peptide and fibronectin (or any other known ECM protein), but still it competes with RGD for binding to the receptor. However, the interaction may be limited to the human integrin since the peptide was not recognized by mouse or hamster α5β1 (52, 54).

Several different approaches have been taken in order to localize ligand binding sites in α5β1. One such approached was the use of phage display screening of peptide libraries for binding to the FNIII-10 fragment. The peptide sequence WDDG/LWL (55), which is similar to a conserved region in RGD-binding β subunits (135-140 in β1; 124-129 in β3), was found to be enriched in this screening. Furthermore, this sequence is located within the RGD/Me^{2+} binding 14 amino acid peptide of β3 (see above, Fig. 3) which was identified by an independent method (55) (see further under αIIbβ3). Cyclic WDDGWL peptide was found to bind directly to RGD peptides; this interaction was independent of cations (55), confirming the result with the D119A mutant of $\beta3_{118\text{-}131}$. Both activating and inhibiting monoclonal antibodies have been mapped to a small region of β1 (amino acids 207-218) close to the above suggested ligand binding site (56). A sharp and flexible turn is predicted to form within this region by the sequence TNKG (amino acids 206-209). The likely contribution of the α subunit to the ligand recognition has been investigated by systematic mutagenesis within an α5 segment corresponding to a region in α4 in which epitopes for several inhibiting antibodies against α4β1 are located (Fig. 4). Ala-scanning of the third N-terminal repeat of α5 showed that mutations Y186A, F187A, and W188A within a predicted β-turn inhibit ligand binding (57). The strongest effect was obtained by the mutation of F187 (corresponding to Y187 in α4, see below). It is yet not known if these mutations indicate the location of an important contact surface with fibronectin, or if they prevent ligand binding indirectly due to conformational alterations of the receptor.

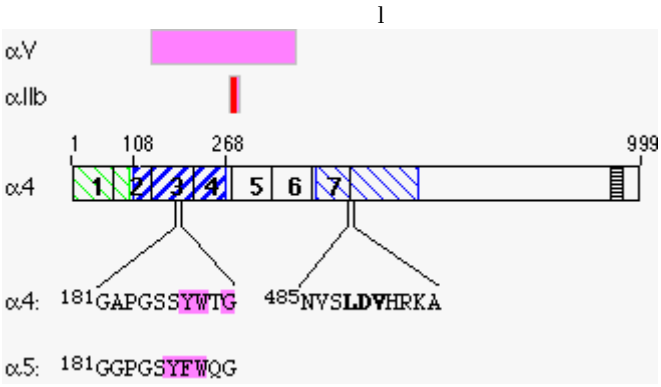

Figure 4. Schematic illustration of the integrin subunit α4. The numbers above the bar refer to amino acids starting from the N-terminus of the mature protein. The squares numbered 1-7 represent repeating sequences of ~70 amino acids which are present in the N-terminus of integrin α subunits. The last three, or in some subunits four, of these repeats each contain a putative divalent cation binding motif. The horizontal black stripes represent the transmembrane part. The epitopes for antibodies which inhibit ligand binding have been mapped to the region marked with bold blue lines. Within this region, the amino acids shaded in pink have been demonstrated to be important for the ligand binding ability of the receptor. Analogous results were obtained for the corresponding residues in α5. The location of the cross-linking regions in αV for RGD peptides and in αIIb for fibrinogen γ-chain 12 amino acid peptide, respectively, are indicated by the pink boxes above α4. The isolated red region of αIIb has been shown to bind directly to fibrinogen via the γ-chain peptide. Antibodies which induce homotypic aggregation of lymphocytes bind to the N-terminal part (thin green lines), while epitopes for antibodies which can block this activity are located in the region marked with thin blue lines. An LDV sequence which may be the binding site for a homotypic receptor interaction is located within this region.

Integrin αIIbβ3: Apart from platelets, in which αIIbβ3 is the major cell surface protein (29, 58), this integrin has only been detected on megakaryocytes (59). Its most important function is to bind fibrinogen during thrombus formation. However, the recognition of fibronectin and several other RGD-containing proteins is probably also physiologically relevant for haemostasis (30).

Both the RGD site and the synergy site DRV**PHSRN**SIT contribute to the binding of fibronectin to αIIbβ3 (60, 61), resembling the binding to α5β1. However, the direct binding of the isolated synergy region can only be demonstrated for αIIbβ3, even though the interaction is ~30-fold weaker compared to RGD peptides (60). Each one of the peptides can efficiently inhibit the binding of fibronectin (or fibrinogen) to isolated receptor or to platelets. Interestingly, the two fibronectin peptides cross-inhibit each other in binding to αIIbβ3 (60), but still they appear to have separate binding sites (see below). A possible explanation for these findings would be that the integrin acts as a closing trap after triggering at either of the two ligand-binding sites. According to this model the unoccupied site would become closed as a result of the conformational rearrangements induced by binding of one of the peptides, while the corresponding event may result in cooperative binding at both sites when the intact fibronectin molecule is available.

Since short RGD peptides bind with higher affinity to αIIbβ3 and αVβ3 than to other integrins, it has been possible to use the peptides in cross-linking experiments to localize binding sites within these receptors. The RGD-containing peptides were found to cross-link mainly to amino acids 109-172 in β3 of αIIbβ3 (62), and to 61-203 in β3 of αVβ3 (63). Subsequently, the synthetic peptide $\beta3_{118-131}$ was identified as an RGD-binding site (46). This region also contains the DXSXS motif which is involved in coordination of one Me^{2+}(46). Based on molecular modeling and mutagenesis by Ala-scanning, two additional amino acids, D217 and E220, were suggested to contribute to the ion binding site. Mutation of either one of these five oxygenated amino acids results in inactivation of αIIbβ3 (49). The importance of the region containing D217 and E220 was further demonstrated when overlapping synthetic peptides spanning the N-terminal 288 amino acids were tested for the ability to inhibit binding of fibronectin and fibrinogen to αIIbβ3 (64).

The fact that αVβ3 does not recognize the synergy site of fibronectin suggests that the a unit in αIIbβ3 contains an important contact surface for the binding of that region. There is no direct data available to support this assumption, but the information obtained for the interaction of fibrinogen with αIIbβ3 may be relevant for this issue. Fibrinogen binds to the receptor via RGD site(s) as well as by a 12 amino acid peptide from the C-terminus of the γ chain (a non-RGD site). The γ chain

peptide was found to crosslink mainly to amino acids 294-314 in repeat 5 of αIIb (65). Further, the peptide $\alpha IIb_{296-306}$ binds directly to fibrinogen and inhibits binding of fibrinogen to αIIbβ3 (66). It remains to be tested if $\alpha IIb_{296-306}$ is the binding site also for the fibronectin synergy peptide. The peptide represents the second metal ion binding site in αIIb, corresponding to the first in α4 (amino acids 278-297). It has been speculated that the second cation which is displaced from αIIbβ3 upon ligand binding would be released from $\alpha IIb_{296-306}$ (see above) (46).

Integrin α4β1: The function of α4β1 has been studied mainly in white blood cells, but it is expressed also by several types of adherent cells (67-69). It can mediate cell-cell contacts as well as cell-matrix contacts through the two known ligands, VCAM-1 and fibronectin, respectively (34). Both these interactions are important for the process of leucocyte extravasation, and thereby for a number of immunological and inflammatory events. Activating signals are required to induce strong binding of α4β1 to VCAM-1 or fibronectin, but even in an unactivated state α4β1 is able to mediate a weak binding to VCAM-1, by which lymphocytes can roll along activated endothelial cells (70, 71).

α4β1 interacts primarily with the III_{14}-V region in fibronectin. Among the three known binding sites within this region, CS1 is predominant in the fibronectin splice variants where it is present (32). CS1 and CS5 cross-inhibit each other for binding to α4β1 (35), but the affinity of recombinant fibronectin fragments for the receptor is higher when both of the sites are included (32), suggesting that they are not competing for the same binding site. The results thus resemble those obtained for the interactions of the RGD and synergy sites with α5β1 and αIIbβ3, although the combined effect of CS1 and CS5 is less dramatic and therefore said to be additive rather than synergistic. H1 was reported not to inhibit the binding of CS1 to α4β1 (72), but higher concentrations of H1 have to be tested, as well as the reverse inhibition experiment, before a firm conclusion can be made. Obviously, determination of the structure of the III_{14}-V region at atomic resolution would greatly increase our understanding of how the multiple cell-binding sites may cooperate.

α4β1 can be induced to recognize also the RGD region of fibronectin (73). The interaction is detectable only in the presence of integrin activating antibodies, hence its physiological relevance remains to be determined. Nevertheless, under these conditions the binding of large RGD-containing fibronectin fragments to the integrin can be inhibited by short peptides with the efficiency decreasing in the order CS1>GRGDSPC>H1 (73).

Monoclonal antibodies against α4 which inhibit binding to CS-1, as well as VCAM-1, map to amino acids 108-268 (the so called B epitopes) (74, 75). Ala-scanning mutagenesis within this region revealed that Y187A, W188A, and G190A inhibit binding of CS-1 and VCAM-1 (57). These amino acids are part of a predicted β-turn between two β-sheets in the third N-terminal repeat of α4 (76). This repeat lacks cation binding motifs. Mutations in the corresponding turn in α5 also inhibits binding to fibronectin (see above) (57).

Another activity of α4β1 which is inducible by certain monoclonal antibodies against α4 is aggregation of lymphoid cells (77-79). These monoclonal antibodies have all been mapped to the N-terminal 100 amino acids (A epitopes) (74, 75). Antibodies capable of blocking the induced aggregation bind to amino acids 422-606 of α4 (C epitopes) (74, 75). Since there are no known ligand for α4β1 on lymphocytes, the possibility of homotypic binding of this receptor to itself has been suggested. In support of this concept, α4β1 on lymphocytes was shown to bind to an LDV-containing region of the isolated α4 chain ($LDV_{488-490}$ located within the C epitope region) (80). Although the data are convincing, they raise several questions: In which physiological situations would homotypic α4β1 interactions occur? Could the interactions reflect lateral interactions between integrins in the same cell (allowing high avidity binding to an unknown ligand) rather than binding between integrin molecules on different cells? Is homotypic binding of α4β1 restricted to certain cells (*i.e.* lymphocytes), since expression of α4β1 in K562 (erythroleukemia) or CHO cells did not induce aggregation of the cells (81). Is the ability of homotypic interaction unique for α4β1 or shared by other integrin(s)?

Integrin α3β1: The normal distribution of this integrin *in vivo* is limited mainly to epithelial cells in the skin, endothelial cells of the digestive tract, and kidney mesangial cells (82-85). In contrast, it is expressed by most cells in culture and by many tumors *in vivo* (86, 87). Laminin 5 (kalinin) appears to be an important ligand for the receptor (88, 89), while a weak binding to fibronectin and several other ligands has been observed only under certain conditions (82, 90-92). Further, α3β1 is unable to mediate initial cell adhesion to any of these proteins except for laminin 5, indicating that it does not function as a fibronectin receptor in intact cells (93, 94). The issue is confused by the fact that α3β1 localizes to focal contacts in cells seeded on fibronectin; however, this localization was shown to occur independently of which protein the cells adhered to (85). α3β1 has therefore been suggested to serve as a secondary receptor with post adhesion functions (*e.g.* signaling for growth?) (85). It is unknown whether low affinity interactions with

fibronectin or other matrix molecules would be of any importance for such functions.

Integrin α8β1: This receptor is expressed mainly in epithelial cells, smooth muscle cells, myofibroblasts, and embryonic neural cells (95, 96). It has been shown to bind to RGD sites in fibronectin and vitronectin (97). In addition, tenascin-C has been reported to bind to α8β1 via both RGD-dependent and RGD-independent interactions (97). α8β1 is able to mediate cell adhesion and neurite outgrowth on these ligands (98, 99). Several other RGD-containing proteins, collagens, and CAMs were not recognized by α8β1 (97).

Integrin αVβ1: αVβ1 is present on the surface of some cell lines, and appears to recognize fibronectin as its major ligand (100, 101). Although the subunits αV, β1, and β3 are expressed simultaneously by many cells, the combination αVβ1 is seldom detected. This raises the question of how the formation of multiple integrins is regulated inside a cell. Is it just the relative amounts of the different subunits that determine which heterodimeric receptors are formed (simple competition on equal terms), or do the α and β subunits have some preferred partners?

Integrin αVβ3: This integrin is abundantly expressed by many cultured cells, but its distribution *in vivo* is restricted mainly to activated endothelial cells, osteoclasts, and tumor cells (36). It has been demonstrated to be important for angiogenesis and is therefore a potential target for inhibition of tumor growth (102-104). αVβ3 was the first vitronectin receptor to be identified (105), but later it was found to bind also fibronectin and several other cell adhesion proteins (28, 106, 107). In all cases the RGD motif in the ligands is of critical importance for binding of αVβ3. As discussed above, αVβ3 does not require the synergy site of fibronectin for stable interaction (60, 108).

Integrin αVβ6: αVβ6 is expressed by epithelial cells during development and wound healing, and in many epithelial tumors (109). In this context it is interesting that the C-terminal 11 amino acids of the cytoplasmic part, which is unique to β6, contains a proliferation promoting activity (110, 111). The cytoplasmic tail of β6 also contains three regions required for localization of αVβ6 to focal contacts (the membrane proximal region and two NPXY motifs) (110); the corresponding regions in β1 and β3 share the same function.

Fibronectin appears to be the main ligand for αVβ6 (112), but a weaker interaction with tenascin-C has also been described (113). αVβ6 binds fibronectin via the RGD site, and like αVβ3, it does not require the synergy site in fibronectin (114).

Integrin α4β7: This integrin is expressed on subsets of lymphocytes (115). Similar to α4β1, it binds to the III_{14}-V region in fibronectin, VCAM, and the LDV site in the α4 subunit (116). In addition, it recognizes MAdCAM-1, an interaction which is important for homing of specific lymphocyte populations to mucosal sites (117).

Integrin α?β8: The β8 subunit is expressed mainly in brain, spinal cord, kidney, and embryonic muscle (118). The sequence of β8 is rather different from other integrin subunits; for example, the cytoplasmic domain has no similarity to the other β subunits (118). The α subunit(s) associated with β8 unit has not yet been identified for the fibronectin receptor. αVβ 8 can form in β8 transfected 293 cells, but it does not bind fibronectin (118, 119). Antibodies specific for the β8 subunit co-immunoprecipitated one or more α components from sensory neurons (not αV) having unreduced Mr ~110-120kD in SDS-PAGE. The antibody could inhibit neurite outgrowth from these cells seeded on either fibronectin, laminin 1, or collagen IV (120) Since this is an unusual combination of ligands for integrins, it appears likely that 8 can combine with several different αV.

Besides mediating cell adhesion, all integrins can participate in one or more of the

outgrowth, organization of the cytoskeleton and focal contacts, and signaling which affects gene expression,

new extracellular matrix is an activity so far only described for fibronectin binding integrins. In contrast *e.g.* collagens,

spontaneously *in vitro*
Instead, the reaction occurs on the cell surface and involves interaction of fibronectin with integrins and

major ECM components such as collagens, fibulins, and proteoglycans (121, 122) may be dependent on interactions with fibronectin for their organized

reaction of soluble fibronectin protomers to insoluble networks is central for matrix formation. The general

fibronectin polymerization (123, 124) further highlights the importance of the process.

α5β1 was the first integrin identified to be involved in fibronectin network formation. It localizes to the sites where fibronectin fibrils are in contact with the cell , and antibodies to α5 or β1 can inhibit the polymerization in fibroblast cultures (126). Furthermore, overexpression of α5 in CHO cells increases the deposition of fibronectin in the matrix (124). However, mouse embryos deficient in the α5

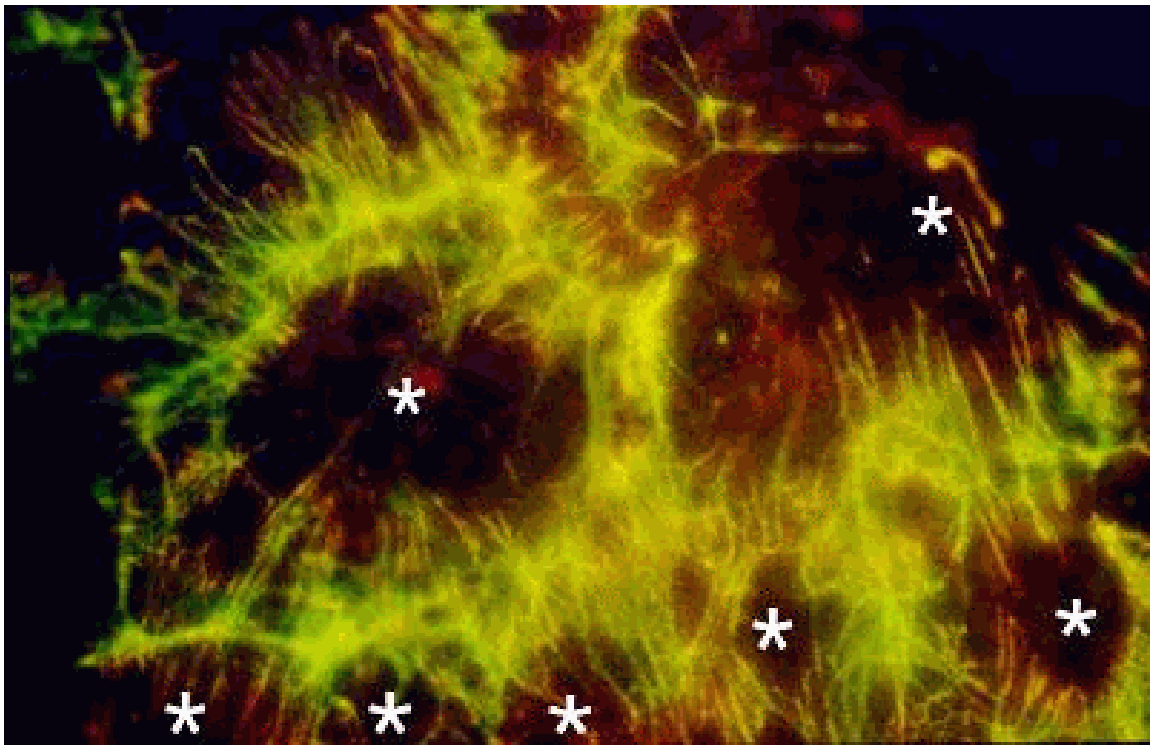

Figure 5. Double immunofluorescent staining for fibronectin (green) and integrin subunit α5 (red) in a culture of GD25-β1A cells (107), grown on coverslips for 5 days before fixing and staining. Cells are marked with asterisks (*). Note that most fibrils either seem to be stretched between two cells or are positioned at cell-cell borders, and that the ends of the fibrils colocalize with α5. The staining was performed as described (107).

gene still contained extracellular fibronectin networks (127), indicating that also other integrins could promote the polymerization process. At present, αVβ3 (107) and αIIbβ3 (128) have been shown to have this potential, although αVβ3 was markedly less efficient than α5β1, and αIIbβ3 probably never serves this function *in vivo*. Expression of αVβ1 (101) or α4β1 (129) in CHO cells did not promote fibronectin polymerization, illustrating that mere binding of fibronectin to the cell surface by any integrin is not sufficient. A connection of the β subunit to actin filaments is known to be required for fibronectin fibril formation on the cell surface (128, 130). Other factors which may influence whether an integrin has the fibril promoting activity include the recognition site preference in the fibronectin molecule (RGD, LDV, etc.) and the receptor-ligand binding affinity.

The polymerization process can be separated into two phases, initiation (nucleation) and extension, both of which are poorly understood. It has been reported in one study that the fibrils grow at only one end, indicating that they are polarized (131). Alternatively, the direction of fibril growth may reflect the migration of the cell. It is not known if new protomers are added to the fibril at the cell surface, or somewhere else along the fibril. The issue is further confused by the fact that the fibrils often appear to be anchored at both ends to cell surfaces, either on one cell or on two different cells (Fig. 5). The inability of monomeric fibronectin chains to become incorporated into the fibrils (132) may be relevant in this context.

Several regions in fibronectin are involved in one or the other phases of polymerization, through binding to cell surface components or to a neighbouring fibronectin molecule (Fig. 6). Binding of the RGD region to integrins may be the initial event which triggers the subsequent reactions (126), possibly by induction of an altered fibronectin conformation. The inability of recombinant fibronectin lacking the RGD motif to initiate fibril formation supports this idea (133). However, it is unclear whether fibronectin in its soluble folded conformation can bind to α5β1. On hepatocytes α5β1 was found to recognize the protein only after treatments which are known to unfold fibronectin (*i.e.* interaction with a specific collagen fragment or heparin, limited proteolytic cleavage) (17). Furthermore, fibronectin fragments rather than the intact protein are commonly chosen for affinity isolation of α5β1 (51, 108, 134). It can be speculated that folding at the suggested hinge between III_9 and

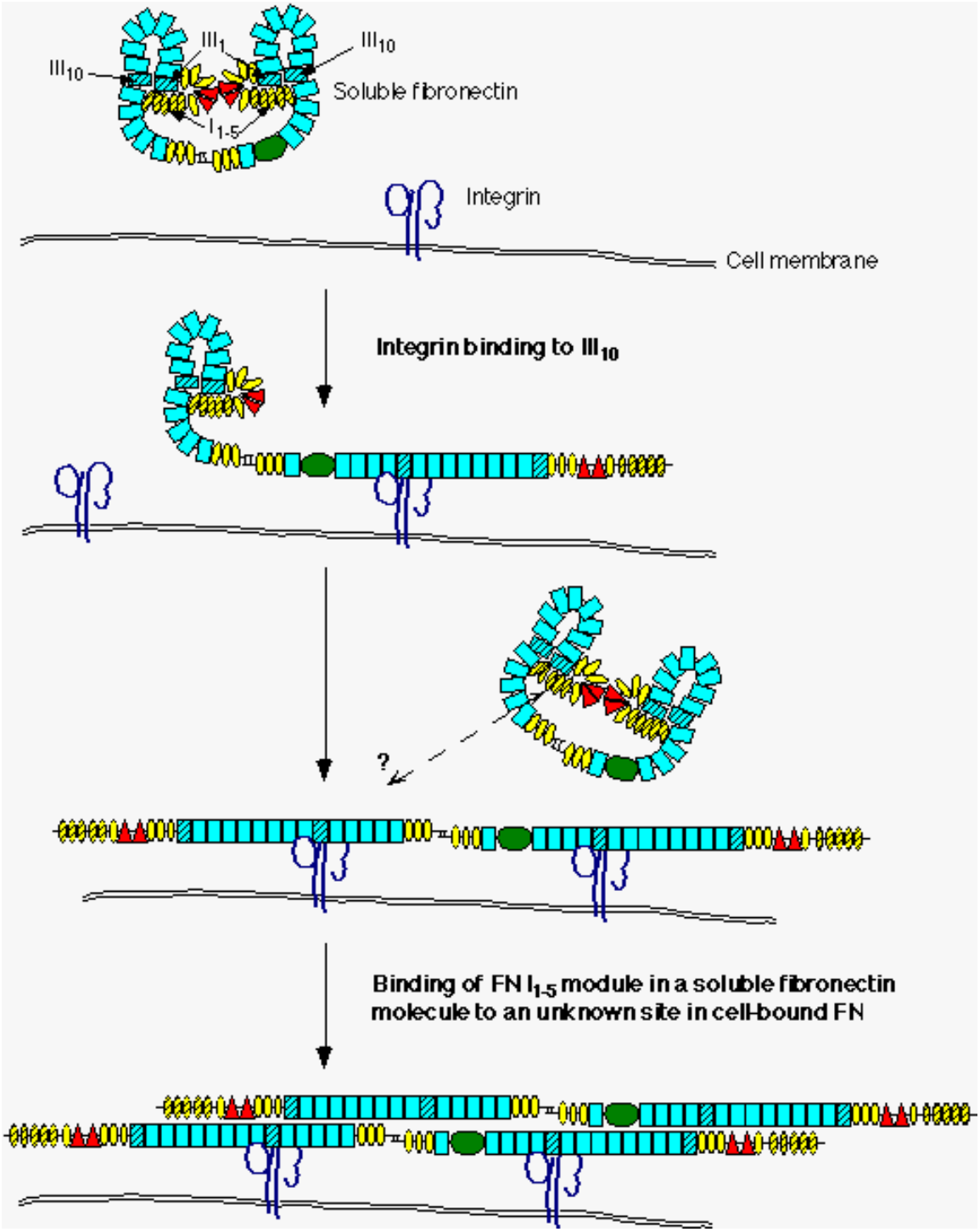

Figure 6. Hypothetical model of the initial events in fibronectin polymerization. A soluble fibronectin molecule is bound to the surface of a cell via III_{10} to fibronectin-binding integrins. The interaction induces a conformational change in the fibronectin molecule which becomes elongated. The cell bound fibronectin exposes binding sites (unidentified) for $I_{1\text{-}5}$ in another soluble fibronectin molecule. This interaction will "open up" the second fibronectin molecule, and so on. Pulling force from the actin filament system may be required for the postulated conformational changes. Domains in fibronectin which may be involved in interactions with other fibronectin domains are marked with black stripes.

III_{10} in soluble fibronectin (31) would prevent $\alpha5\beta1$ from reaching both the synergy site and the RGD loop. Thus, modulation of the fibronectin structure by some other interaction may preceed its binding to $\alpha5\beta1$.

The isolated N-terminal I_{1-5} domain has been shown to bind cell layers at sites of fibronectin fibril formation, and to inhibit further incorporation of intact fibronectin molecules (135, 136). Deletion or disruption of this functional unit in recombinant fibronectin prevents incorporation of the protein into fibrils (132, 137). The N-terminal domain has been suggested to bind to a non-integrin "matrix assembly receptor" (135), but the nature of the receptor still remains unknown (138). Instead, accumulating evidence indicates that I_{1-5} binds either to conformationally altered fibronectin (139, 140) or to a site on $\alpha5\beta1$ which becomes exposed after binding RGD ligands (141). In either of the latter two models, I_{1-5} would be involved in fibril extension rather than initiation. The postulated conformational changes could result directly from the interactions or from a pulling force of the actin filament system. The importance of the cytoskeleton has been demonstrated by use of cytochalasin B, and fibronectin fibrils running between two cells appear to be under tension as indicated by immunofluorescent staining (Fig. 5).

A cryptic site in III_1 can bind to I_{1-5} (139, 140) and a similar interaction was described between unfolded III_{10} and III_1 (142). At present it is not known which of these potential interactions mediate inter- and/or intramolecular bridging between fibronectin domains. The difficulties in studying these events is a major reason why the exact mechanism of the polymerization process still is unsolved.

3.6. *In vivo* functions of fibronectin and its integrins receptors

During embryogenesis, fibronectin appears before or at the onset of gastrulation in all vertebrates examined, and it is abundant at times and sites of cell migration: during gastrulation, neural crest cell migration and the migration of primordial germ cells (143). Alternative mRNA splicing is used during development as a mechanism to create different forms of fibronectin within the extracellular matrix by inclusion or exclusion of the III_A, III_B, and V segments (144-146). The expression of integrins has also been demonstrated to be developmentally regulated, where some of the fibronectin receptor subunits are continuously expressed, while others are not (147). Injection of antibodies to fibronectin or RGD-containing peptides inhibits gastrulation of several species, indicating that the interactions between fibronectin and integrins are important during that particular stage of development (148-151). To investigate the role of fibronectin and each of its receptors, the genes of both fibronectin and several integrin subunits have been knocked out by homologous recombination (37, 38, 152). Up to now, disruption of the genes for five α-subunits ($\alpha3$, $\alpha4$, $\alpha5$, $\alpha8$ and αV) (127, 153, 154) and three β subunits ($\beta1$, $\beta6$ and $\beta7$) (155-159) of fibronectin binding integrins has been reported. Since several of the knock-outs are lethal at embryonic stages, the generation of chimeric mice have been necessary in order to investigate the effects on later stages of development and in adult animals. To generate chimeric animals, embryonic stem (ES) cells in which both alleles of the gene of interest have been disrupted are injected into a normal blastocyst. The resulting mouse will have a mixture of cells which are normal and which are homozygous for the disrupted gene. This enables the investigation of the contribution of the null-cells in different organs and in different processes. Further information about the function of the disrupted gene product can be obtained by *in vitro* studies of the ES cells. For example, the differentiation process of the mutated ES cells into specific cell types can conveniently be followed and manipulated in cell cultures.

Fibronectin: Mouse embryos lacking fibronectin (fibronectin-null) die at embryonic day 8.5, and they have defects in the development of mesoderm, neural tube and blood vessels (152, 160). Although fibronectin is expressed in normal preimplantation blastocysts, the fibronectin-null blastocysts hatch and implant into the uterine wall. They initiate gastrulation and form mesoderm in the complete absence of embryo derived fibronectin. Nevertheless, the presence of fibronectin at these early stages is believed to be important, since the oocyte alone is probably contributing with enough maternal fibronectin for these processes to occur. From embryonic day 8.0 and onwards the mutant embryos develop deformities and deteriorate during day 10-11 of gestation. Fibronectin-null embryos show shortened anterior-posterior axes, fail to develop certain mesodermally derived structures like notochord and somites, and develop abnormal heart and blood vessels, all probably a result of a deficit in the mesodermal layer. The lack of notochord and somites has later been shown to be a result of fibronectin being critical for the organization or maintenance of the notochord precursor cells and for the condensation of precursor cells into somites (160). Neural folds are formed in the absence of fibronectin, while the neural tube becomes kinked. Primitive red blood cells do develop in the fibronectin-null embryos, while blood vessels do not, strongly suggesting a role for fibronectin in vasculogenesis but not in hematopoiesis.

Since there are several receptors for fibronectin, the total effect of the fibronectin-null mutation is likely to be made up of separate effects due to lack of binding between fibronectin and its individual receptors. Therefore, studies on knock-outs

of each of the fibronectin receptor subunits make it easier to determine where the different receptors are active, what functions they have, and what their roles are during embryonic development.

β1: The β1 subunit can dimerize with at least 10 α subunits, of which five have been shown to bind fibronectin (α3β1, α4β1, α5β1, α8β1, and αVβ1). The homozygous β1-null embryos develop normally to the blastocyst stage, implant, but die at embryonic day 5.5 (155, 156, 158). The β1-null throphoblast cells are able to invade the uterine stroma and survive longer than the inner cell mass, suggesting that the inner cell mass requires β1 integrins for survival while the trophoblasts do not. Analysis of the chimeric embryos demonstrated the presence of β1 integrin-deficient cells in all germ layers, indicating that β1-null cells can differentiate and migrate in the environment provided by most normal tissue. For example, chimeric mice show patches of myocytes that are β1-null in cardiac muscle, and myotubes show some β1-null contribution as a result of fusion between wild-type and mutant myoblasts into mixed myotubes. However, lack of the β1 subunit markedly retards the formation of cardiac and skeletal muscles (161, 162) when the differentiation from ES cells was studied *in vitro*. In conflict with previous reports (68, 163), studies using *in vitro* and *in vivo* differentiated myoblasts have proven that the β1 integrin is not necessary for sarcomer or myotube formation (162), events which represent late stages of the differentiation process. Migration of neuronal cells as well as neural crest cells can occur in the absence of β1 integrins. In contrast, the chimeric mice lack β1-null cells in blood and in haematopoietic organs such as spleen, thymus and bone marrow as a consequence of the inability of β1-null cells to invade the fetal liver (164). The β1-null embryos die before the fibronectin-null embryos. The reason for this can be that β1 containing integrins that bind ligands other than fibronectin are important at earlier stages, before the β1/fibronectin interactions take place. Alternatively, it can be due to the different life-times of the β1 and the fibronectin maternal mRNAs provided by the oocyte.

α5: The α5 subunit is found only in combination with β1. The knockout of the α5 subunit resulted in nearly the same defects as the fibronectin-null mutation did (127), suggesting that the majority of the defects seen in the fibronectin-null embryos probably are due to the lack of α5β1/fibronectin binding. The α5 mutant embryos start to show defects by embryonic day 8.5 and die around day 10-11. The α5-null embryos have defects in the posterior trunk and yolk sac mesodermal structures, suggesting a role for α5β1 integrin in mesoderm formation, movement or function. The morphogenesis in α5-null embryos seems to extend further compared to the fibronectin-null embryos, including formation of notochord, somites and heart, considerable development of the brain, optic and otic anlagen and formation of branchial arches. The anterior part of the α5-null embryo develops relatively normally up to about the tenth somite. However, the posterior somites are absent, the paraxial mesoderm are defective, and the neural tube becomes kinked as it does in the fibronectin-null embryos. The α5β1 defective embryo develops a heart but shows defects in vascular development, although again the defects due to the absence of α5β1 are less severe and more limited to the posterior part than those due to the absence of fibronectin. It seems that α5β1 is required for proper formation and maintenance of blood vessels, while other fibronectin receptors are involved in initial steps of vasculogenesis (127).

α4: The α4 integrins (α4β1 and α4β7) are both capable of binding fibronectin as well as VCAM-1. The α4-null embryos die at embryonic day 11 due to the lack of a functional placenta (154). The allantois fail to fuse with the chorion during placentation and the development of the epicardium and the coronary vessels are impaired leading to cardiac haemorrhage. However, the α4 subunit seems not to be essential for the formation of either cardiac or skeletal muscle (154, 165). Studies of the VCAM-1 knockout suggest that these α4-null phenotypes are a result of abolished α4/VCAM-1 interactions rather than α4/fibronectin interactions (166, 167). Chimeric mice were also used to study blood cell development (168). During fetal life, T-cell development is α4 independent, but after birth further production of T-cells becomes α4 dependent. Precursors for both T- and B-cells require α4 integrins for normal development within the bone marrow, while monocytes and natural killer cells can develop normally without α4 integrins.

β7: The β7 subunit can dimerize with α4 and the integrin α4β7 is expressed on a subset of lymphocytes together with α4β1. It has been shown that lack of β7 leads to an impaired formation of the gut-associated lymphoid tissues (159). This is probably a result of inability of the lymphocytes to adhere to the endothelium and subsequently to populate the gut-associated lymphoid tissues. Whether this is a result of disrupted interactions of α4β7 with VCAM-1, MAdCAM, or fibronectin is not known.

α3: The α3-subunit can dimerize with the β1 subunit, and α3β1 is a receptor for laminin 5 and possibly for fibronectin. The homozygous α3 mutant mice die during the first day after birth (153). The α3 mutants have several kidney and lung abnormalities, consistent with the epithelial expression in these organs of normal mice. The tubules in the medullary region of the kidney are dilated, and the bronchial

airways of the mutant lung are wider than normal. The mutant kidney cells have a greatly decreased number of foot processes along the glomerular basement membrane, and the basement membrane is wider and more disorganized when compared with the normal kidney. Since a recent report indicates that α3β1 does not really bind fibronectin but that it rather has a secondary function (85) that may be important for fibronectin assembly (169), further investigation is needed to determine if the defects in the α3-knockout is a consequence of lack of binding to its major ligand laminin 5, rather than a fibronectin related effect.

α8: The α8β1 integrin binds fibronectin and is expressed in adult smooth muscle and epithelia, and in mesenchymal and neural cells during development. The α8 gene has been knocked out, but the original results have not been published yet. Apparently, lack of α8 results in defect development of the kidney, indicating a role for α8β1 in kidney morphogenesis (37, 38).

αV: The αV subunit can dimerize with five β subunits, three of which are able to bind fibronectin (αVβ1, αVβ3, and αVβ6). The αV subunit has been knocked out, but the original data has not yet been published. Apparently, the mouse die on the first day after birth, probably as a cause of vascular haemorrhage (37, 38). The αV containing integrins have a wide range of ligands in addition to fibronectin, and this complicates the interpretation of the results with regard to fibronectin and its functions.

β3: The β3 subunit has, to our knowledge, not been knocked out. However, the lack of, or mutations in, β3 lead to bleeding disorders in man (Glanzmann's disease) due to the impaired ability of the platelets to aggregate at injury sites. Surprisingly few other abnormalities are observed in these patients.

αIIb: The αIIb subunit has not been reported knocked out (however, see β3).

β6: The integrin αVβ6 is the only integrin expressed exclusively in epithelial cells and has been found to bind fibronectin and tenascin-C (112, 113). The expression of αVβ6 is rapidly induced following epithelial injury while only barely detectable on normal adult epithelia. During embryogenesis, αVβ6 is expressed in lung, skin, and kidney. The β6-null mice have juvenile baldness associated with degenerated hair follicles and infiltration of macrophages into the skin, and accumulation of activated and cytokine producing B- and T-cells around conducting airways in the lungs (157). The lungs show enhanced sensitivity to acetylcholine, a hallmark feature of asthma, suggesting that αVβ6 participates in the modulation of epithelial inflammation. On the other hand, the β6-null mice were capable of healing cutaneous wounds, which had been suggested to be αVβ6 dependent. Further studies are needed to determine whether it is fibronectin or tenascin-C that is involved in these conditions, but the lack of the corresponding abnormalities after disruption of the tenascin-C gene strongly indicate that the interaction between αVβ6 and tenascin-C is of minor importance (170).

Taken together, these knock-out experiments of both fibronectin and its integrin receptors clearly demonstrate that these proteins are of vital importance for the organism. They also show that the different integrins have distinct functions and distribution, and that they in most cases do not compensate for each other. Nevertheless, αV integrins have been found to be able to compensate for α5β1 integrin function with regard to adhesion to fibronectin and fibronectin matrix assembly *in vitro* (107, 165). The other important aspect of these knock-out investigations is that they complement the earlier antibody or peptide inhibition studies, by providing new and often contradictory conclusions of the *in vivo* functions of fibronectin and integrins. They also show that a protein can be present while not necessarily have a critical function at that particular location at that particular time. Further studies using tissue specific knock-outs, knock-ins, and disruption of one splice variant at the time, will hopefully give even more detailed and interpretable information about the *in vivo* roles of fibronectin and its integrin receptors.

3.7. Perspectives

Integrin α5β1 was isolated in 1985 as the first fibronectin receptor (51) and its cDNA sequence was reported two years later (171). Since then an impressive amount of information on the structure, interactions, and functions of fibronectin and its receptors has accumulated. As convincingly demonstrated by gene knock-out technology, these proteins are of fundamental importance in various physiological situations. Therefore, they will continue to be subject for intense research, and the progress rate will probably be faster than we can foresee. Some of the more immediate goals to reach include information on the three dimensional structures of whole integrins and fibronectin, and understanding of how conformational changes in these proteins are regulated. One area where such information would be of practical use is in development of reagents which could modulate receptor function during treatment of various adhesion-related disorders. These reagents could potentially be designed to act directly on the ligand binding sites, to affect conformation, or to modulate interactions with integrin associated proteins inside or outside of the cells.

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