

Chapter 2

Fibronectin and Other Adhesive Glycoproteins

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Abstract Cells adhere to the extracellular matrix through interaction with adhesive extracellular matrix glycoproteins, including fibronectin, laminins, vitronectin, thrombospondins, tenascins, entactins (or nidogens), nephronectin, fibrinogen, and others. Most adhesive glycoproteins bind cells through cell surface integrin receptors in conjunction with other cell surface receptors, such as dystroglycans and syndecans, and interact with other extracellular matrix proteins to form an intensive matrix network. Interactions between cells and the extracellular matrix may mediate many cellular responses, such as cell migration, growth, differentiation, and survival. Cells receive and respond to signals from surrounding extracellular matrix, and in turn, modulate surrounding extracellular matrix through control of matrix assembly. This chapter discusses the adhesive glycoproteins and focuses on the interaction between integrins and adhesive glycoproteins.

2.1 Introduction

The interaction between cells and glycoproteins of the extracellular matrix mediates cell adhesion, migration, growth, differentiation, and survival of adherent cells. Each of these glycoproteins has distinct functional domains or polypeptide sequences to bind specific cell surface receptors, such as the integrins, dystroglycan, and syndecans; or to interact with other extracellular matrix proteins such as collagens.

Integrins are arguably the most important cell surface receptors that anchor cells to the extracellular matrix. We focus on the interaction between integrins and adhesive glycoproteins in this chapter. We concentrate on two aspects: first, the integrin-binding sequences, especially the dominant integrin-binding residue – aspartate – of each adhesive glycoprotein; second, the relationships between the

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ligand–integrin interaction and the deposition of the ligand in extracellular matrix. The major cell adhesion protein, fibronectin, which interacts with more than ten different integrin receptors, is considered in the greatest detail. Other adhesive glycoproteins, including laminins, vitronectin, thrombospondins, tenascins, entactins, nephronectin, and fibrinogen, are discussed.

2.2 Fibronectin

2.2.1 Overview

Fibronectin was first discovered in 1948 as a contaminant of plasma fibrinogen with insolubility at low temperature and was termed “cold-insoluble globulin” (Morrison et al. 1948; Mosesson and Umfleet 1970). Fibronectin is a high molecular weight dimeric glycoprotein (~450 kDa per dimer) widely expressed by a wide variety of cells in embryos and adult tissues (Hynes 1990; Mosher 1989). Plasma fibronectin is synthesized in the liver by hepatocytes and present in a soluble form in blood plasma at a concentration of around 300 µg/ml. Cellular fibronectin is secreted by fibroblasts and multiple other cell types and is organized into fibrils contributing to the insoluble extracellular matrix. The name “fibronectin” is derived from the Latin word *fibra*, meaning fiber, and *nectere*, meaning to bind. Fibronectin is crucial for vertebrate development, presumably by mediating a variety of adhesive and migratory events. Targeted inactivation of the fibronectin gene is lethal at embryonic day 8.5 in embryos homozygous for the disruption (George et al. 1993). Plasma fibronectin is also important for thrombosis. Conditional fibronectin knockout mice with plasma fibronectin levels reduced to less than 2% of normal have a delay in thrombus formation after vascular injury and defects in thrombus growth and stability (Ni et al. 2003). Fibronectin is organized into a fibrillar network on the cell surface through interaction with cell surface receptors and regulates cell functions, such as cell adhesion, migration, growth, and differentiation (Hynes 1990; Mosher 1989).

2.2.2 Structure of Fibronectin

2.2.2.1 Basic Structure

Visualization of soluble fibronectin by rotary shadowing electron microscopy in the early 1980s revealed two identical and apparently flexible strands (Engel et al. 1981; Erickson et al. 1981). Fibronectin mainly exists as a dimeric glycoprotein, with two similar ~240-kDa subunits covalently linked through a pair of disulfide bonds near the C-terminus. There are three types of repeating modules in each

fibronectin subunit: 12 type I (termed FN1), 2 type II (termed FN2), and 15–17 type III repeats (termed FN3) (Fig. 2.1); accounting for 90% of the sequence. The remaining sequences include a connector between modules ⁵FN1 and ⁶FN1, a short connector between ¹FN3 and ²FN3, and a variable (V) sequence that is not homologous to other parts of fibronectin.

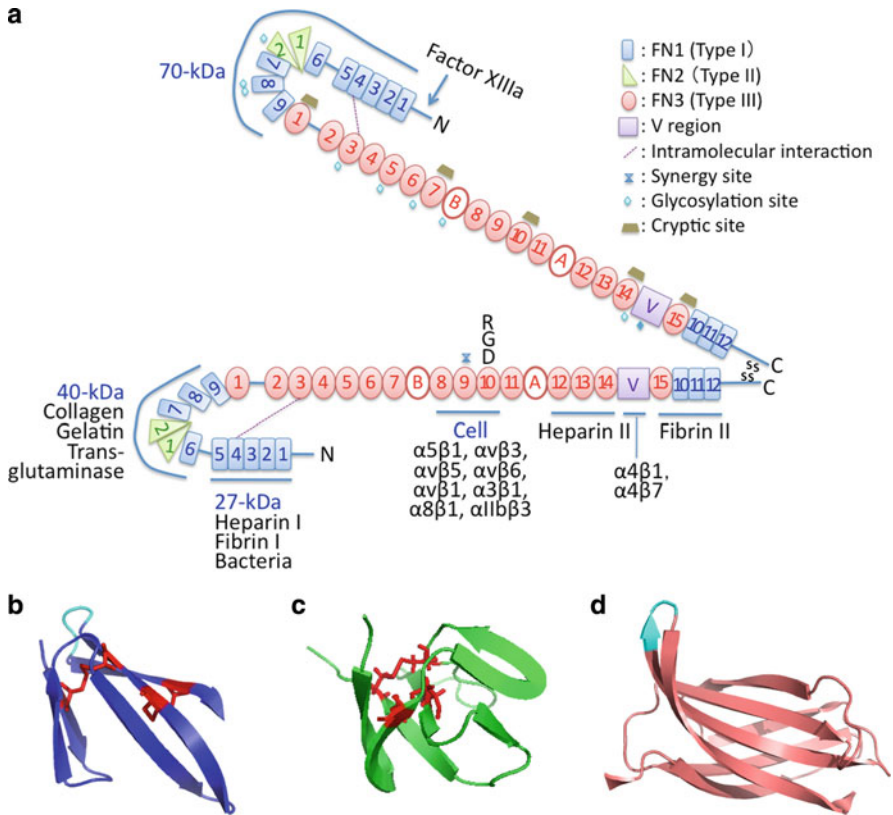


Fig. 2.1 Diagram of fibronectin modular structure and structures of fibronectin modules. (a) Diagram of the modular structure of fibronectin. Each fibronectin dimer is composed of two monomers linked at the C-terminus by a pair of disulfide bonds. 12 type I modules (blue rectangles) termed FN1, 2 type II modules (green triangles) termed FN2, and 15–17 type III modules (salmon ovals) termed FN3. The number of FN3 modules varies due to the presence of ^AFN3 (EDA) and ^BFN3 (EDB) based on alternative splicing. The alternatively spliced V region is shown as a purple square. Proteolytic 27-kDa, 40-kDa, and 70-kDa N-terminal fragments and the protein-binding sites on fibronectin are underlined with receptors listed. (b) The ribbon structure of ⁵FN1 is drawn using PyMOL of structure PDB: 2RKY (Bingham et al. 2008). The cysteine residues and disulfide bonds are shown in red, with other residues shown in blue to match panel a. (c) The ribbon structure of ²FN2 is drawn using PyMOL of solution structure PDB: 1E8B (Pickford et al. 2001). The cysteine residues and disulfide bonds are shown in red. (d) The ribbon structure of ¹⁰FN3 is drawn using PyMOL of solution structure PDB: 1FNF (Leahy et al. 1996). The Arg-Gly-Asp (RGD) residues are shown in cyan

The FN1 module is found only in chordates (Tucker and Chiquet-Ehrismann 2009) (see Chap. 1). It has been noted that the N-terminal sub-domain of the VWF type C module of $\alpha 2$ procollagen shows a structural similarity with the fibronectin FN1 module (O’Leary et al. 2004) and suggested that the VWF type C module, which has been found in a large number of proteins of flies and worms, may be the precursor of the fibronectin FN1 module. Each FN1 module is about 45 amino acid residues long and contains two intrachain disulfide bonds (shown in red in Fig. 2.1b). NMR spectroscopy showed that the FN1 module has compact stacked antiparallel β -sheets enclosing a hydrophobic core with conserved aromatic residues (Baron et al. 1990; Potts et al. 1999). One sheet has two strands (A and B), and the other has three strands (C, D, and E). One disulfide bond, which links two nonadjacent β -strands, connects the first and third cysteines. The other disulfide bond, connecting the second and fourth cysteines, links adjacent β -strands D and E.

FN2 modules are rare and are similar to the kringle domains, which are present in lower organisms besides vertebrates (Ozhogina et al. 2001). Interestingly, FN2 modules are found in matrix metallo-proteinases (Collier et al. 1988). Each FN2 module is approximately 60 residues long with two intrachain disulfide bonds in each repeat. NMR spectroscopy shows that the solution structure of FN2 module consists of several highly conserved aromatic residues, two double-stranded antiparallel β -sheets perpendicular to each other, and four cysteines that form two disulfide bonds connecting cysteines 1–3 and 2–4 (Constantine et al. 1992; Pickford et al. 1997) (Fig. 2.1c). NMR studies identified an interaction between 6 FN1 and 2 FN2 (Pickford et al. 2001), and thus the FN2 modules are thought to cause a departure from a “head-to-tail” arrangement of FN modules (Fig. 2.1).

The FN3 module is found in multiple copies in many other extracellular matrix proteins, cell surface receptors, and cytoskeletal proteins of vertebrates and non-vertebrates (Bork and Doolittle 1992). Each FN3 module is about 90 residues long and lacks disulfide bonds. It consists of two antiparallel β -sheets formed from seven β -strands similar to Ig domains without disulfide bonds (Fig. 2.1d). One β -sheet is formed by four β -strands (G, F, C, and C’) and the other β -sheet is formed by three β -strands (A, B, and E), arranged as a β sandwich to enclose a hydrophobic core (Dickinson et al. 1994a; Dickinson et al. 1994b; Leahy et al. 1996; Main et al. 1992). The β -strands are connected by flexible loops. The main integrin-binding motif Arg-Gly-Asp (RGD) (shown in cyan in Fig. 2.1d) is in one of the flexible loops connecting two β -strands (Dickinson et al. 1994b).

2.2.2.2 Alternative Splicing

One large single gene (~50 kb for human fibronectin) encodes fibronectin in most species (Hirano et al. 1983). Alternative pre-mRNA splicing and various posttranslational modifications result in heterogeneity of fibronectin, with up to 20 variants in human fibronectin (French-Constant 1995; Kosmehl et al. 1996). There are two alternatively spliced segments in fibronectin due to alternative exon usage: extra domain A (EDA) located between the 11th and 12th FN3 modules, and extra domain

B (EDB) between the seventh and eighth FN3 modules (Fig. 2.1a). The nonhomologous variable (V) region between the 14th and 15th FN3 modules, which is subject to exon subdivisions, resulting in five different V region variants in human fibronectin (V0, V64, V89, V95, and V120, with the number standing for the number of amino acid residues in each variant). There is a special type of cartilage-specific splicing [termed (V + C)⁻], with fibronectin lacking in the entire V region through the ¹⁰FN1 module (Burton-Wurster et al. 1999; MacLeod et al. 1996).

Alternative splicing of fibronectin is regulated by cell type, stage of development, and age (Ffrench-Constant 1995; Kornblihtt et al. 1996). Fibronectin isolated from plasma tends to have a lower molecular weight than fibronectin isolated from cell culture, which has resulted in the terms, plasma fibronectin and cellular fibronectin. Plasma fibronectin generally lacks EDA and EDB sequences, and contains a subunit that is V0. Cellular fibronectin is a more heterogeneous group of splice variants with variable presence of EDA, EDB, and V region isoforms. Certain isoforms of fibronectin, especially those containing EDA and EDB modules, are upregulated after wounding, and in malignant cells (Ffrench-Constant 1995).

The EDA module of fibronectin mediates cell differentiation (Jarnagin et al. 1994). Fibronectin containing the EDA module is much better at promoting cell adhesion and spreading than fibronectin lacking the EDA module (Manabe et al. 1997). The presence of EDA module in fibronectin enhances fibronectin- $\alpha 5\beta 1$ integrin interaction and promotes cell adhesion (Manabe et al. 1997). A direct interaction between EDA and $\alpha 9\beta 1$ integrins, however, is critical for lymphatic valve morphogenesis through regulation of fibronectin assembly (Bazigou et al. 2009). Genetically manipulated mice that lacked EDA developed normally, but with a shorter life span, abnormal wound healing, and edematous granulation tissue (Muro et al. 2003), suggesting that EDA is not required for embryonic development but is important for a normal life span and emphasizing the role of fibronectin in organization of the granulation tissue and in wound healing. EDB knockout mice developed normally as well, but with reduced fibronectin matrix assembly (Fukuda et al. 2002). The presence of the EDB module exposes a cryptic binding site in the ⁷FN3 module (Carnemolla et al. 1992). EDB-containing fibronectins are concentrated in tumors and are found at low levels in plasma (Menrad and Menssen 2005). For this reason, tumor therapy research has focused on developing antibodies specific to the EDB module of fibronectin.

2.2.2.3 Posttranslational Modifications

In addition to alternative pre-mRNA splicing, various posttranslational modifications that occur intracellularly during trafficking through the endoplasmic reticulum and Golgi contribute to the heterogeneity of fibronectin. Fibronectin can be glycosylated, phosphorylated, and sulfated (Paul and Hynes 1984). The intrachain and intramodule disulfide bonds of FN1 and FN2 modules are formed in this step as well.

There are seven N-linked carbohydrate chains and one or two O-linked carbohydrate chains per fibronectin subunit (Mosher 1989). Generally, fibronectin contains about 5% carbohydrate although higher levels of glycosylation occur in some tissues (Mosher 1989; Ruoslahti et al. 1981). Nonglycosylated fibronectin is more sensitive to proteolysis than glycosylated fibronectin and has an altered binding affinity to proteins such as collagen, suggesting that carbohydrates stabilize fibronectin against degradation and regulate its affinity to some substrates (Bernard et al. 1982; Jones et al. 1986; Olden et al. 1979). The 40-kDa gelatin-binding domain contains three N-linked glycosylation sites (Skorstengaard et al. 1984), with two sites, Asn497 and Asn511, present in the ⁸FN1 module. Nonglycosylated ⁸FN1 has decreased thermal stability and decreased gelatin-binding activity compared with glycosylated ⁸FN1 (Ingham et al. 1995; Millard et al. 2005).

O-phosphoserine was identified at a concentration of two residues per molecule in human plasma fibronectin (Etheredge et al. 1985). Phosphorylation has also been identified in the carboxyl-terminal region of bovine plasma fibronectin (Skorstengaard et al. 1982). Most of the sulfation of fibronectin occurs at tyrosine residues as tyrosine-O-SO₄, probably in the V region (Liu and Lipmann 1985; Paul and Hynes 1984). It should be noted that the referenced analyses are somewhat dated; application of new mass spectrometric techniques should allow localization of modifications to specific residues and may reveal additional sites of modification.

2.2.3 *Functional Domains*

Fibronectin has important roles in mediating a variety of cell adhesive and migratory activities. Fibronectin binds to cells through cell surface receptors (integrins) and specifically interacts with other proteins, including collagen, fibrin, and heparin/heparan sulfate. The functional domains of fibronectin have been defined by studies of proteolytic fragments and recombinant constructs (Pankov and Yamada 2002).

2.2.3.1 *Integrin Interaction Domains*

Two major sites of fibronectin that mediate cell adhesion are the “cell-binding domain” (⁹FN3–¹⁰FN3) and the alternatively spliced V region (Fig. 2.1a). Fibronectin interacts with many integrins. For example, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu \beta 1$, $\alpha \text{IIb}\beta 3$, $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, and $\alpha \nu \beta 6$ integrins interact with the Arg-Gly-Asp (RGD) sequence in the central cell-binding domain. Integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$, in contrast, recognize the Leu-Asp-Val (LDV) sequence in the V region (Humphries et al. 2006; Leiss et al. 2008). The integrin-binding motifs all contain a critical residue Asp (D), which interacts with a metal in the metal-ion dependent adhesion site (MIDAS) in the integrins (Fig. 2.2). Additional integrin-binding sites are also available in the EDA module, which binds $\alpha 4\beta 1$ or $\alpha 9\beta 1$ integrin (Liao et al.

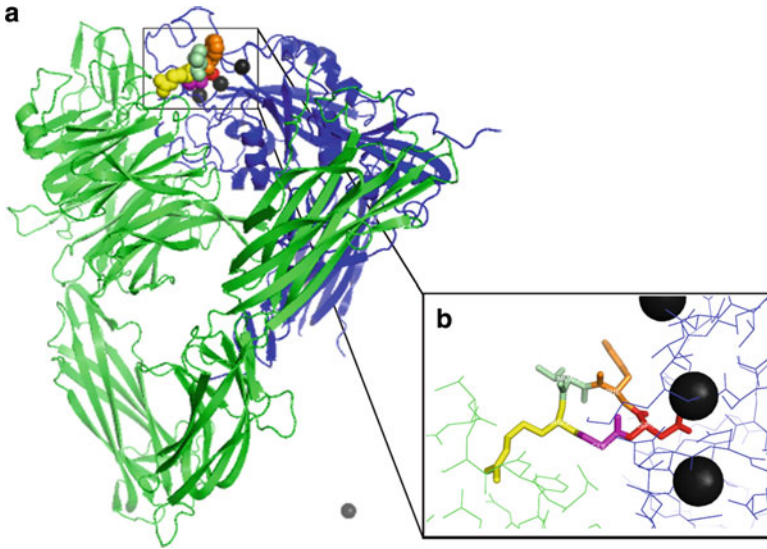


Fig. 2.2 Structure of RGD – $\alpha v\beta 3$ integrin interaction, (a) The ribbon structure of clyco(RGDf-N V) – $\alpha v\beta 3$ integrin interaction in the presence of Mn PDB: 1L5G (Xiong et al. 2002). (b) Ball-and-stick representation of RGD-integrin interaction. αv is shown in green, and $\beta 3$ is shown in blue. The three Mn^{2+} ions are shown in black. The peptide residues R, G, D, f, and MVA are shown in yellow, magenta, red, orange, and pale green, respectively. The aspartate side chain binds the Mn^{2+} in the middle – the MIDAS site

2002); ¹⁴FN3 module, which binds $\alpha 4\beta 1$ integrin through the IDAPS sequence (Pankov and Yamada 2002); and ⁵FN3, which binds activated $\alpha 4\beta 1$ or $\alpha 4\beta 7$ integrin through the KLDAPT sequence (Moyano et al. 1997). Recently, it was demonstrated that the ³FN3 module may mediate cell spreading and migration through interaction with $\beta 1$ integrin(s) combined with specific although unresolved α -subunit(s) in an RGD-dependent manner (Obara et al. 2010). Also, *iso*-Asp-Gly-Arg (*iso*-DGR), spontaneously converted from Asn-Gly-Arg (NGR) by deamidation of asparagine with isomerization of the backbone linkage to the β -position, in ⁵FN1 and ⁷FN1 repeats may interact with $\alpha v\beta 3$ integrins (Curnis et al. 2006).

Major Cell-Binding Domain

The RGD motif, which mediates cell adhesion through interaction with cell surface integrin receptors and widely exists in adhesive glycoproteins such as vitronectin and von Willebrand factor, was first identified in fibronectin in 1983 (Pierschbacher and Ruoslahti 1984). The interesting history of the discovery of RGD motif can be found in an essay written by Ruoslahti (2003).

The RGD motif is essential for development. Site-directed mutagenesis to substitute a Glu (E) for Asp (D) in the RGD motif caused a >95% loss of

cell-adhesive ability (Obara et al. 1988). Mouse embryos in which the RGD motif was replaced with inactive RGE died at embryonic day 10 with shortened posterior trunk and severe vascular defects (Takahashi et al. 2007). Interestingly, changing Arg (R) to Lys (K) to generate peptides with a KGD sequence caused loss of interaction with $\alpha 5 \beta 1$ but the interaction with $\alpha \text{IIb} \beta 3$ was not affected (Scarborough et al. 1993).

In fibronectin, the RGD motif localizes in a flexible loop connecting two β -strands of the $^{10}\text{FN3}$ module, protruding out of the protein structure (Dickinson et al. 1994b) (Fig. 2.1d). The high-affinity interaction of $\alpha 5 \beta 1$ integrin with fibronectin RGD motif requires the synergy site Pro-His-Ser-Arg-Asn (PHSRN) in the $^9\text{FN3}$ repeat (Aota et al. 1994). Crystal structure of a fibronectin fragment of $^7\text{FN3}$ – $^{10}\text{FN3}$ revealed that the RGD loop in the $^{10}\text{FN3}$ module and the “synergy” site in the $^9\text{FN3}$ module are on the same face of $^7\text{FN3}$ – $^{10}\text{FN3}$, presumably enabling simultaneous interaction of both sites with a single integrin molecule (Leahy et al. 1996). Antibody blocking- and epitope-mapping studies with $\alpha 5 \beta 1$ integrin and fibronectin cell-binding domain fragments suggested that the synergy site primarily binds to the α subunit of integrin while the RGD motif binds to the β subunit of integrin (Mould et al. 1997). Mechanical studies showed that there are two forms of $\alpha 5 \beta 1$ -fibronectin bonds: relaxed bonds and tensioned bonds, with the tensioned bonds being required for phosphorylation of focal adhesion kinase (Friedland et al. 2009). It was found that the relaxed bonds only involve the RGD sequence and the tensioned bonds require both RGD and the synergy site. Another recent study using purified integrins found that activated $\alpha \text{v} \beta 3$ integrin could not bind soluble fibronectin, while $\alpha 5 \beta 1$ integrin binds soluble fibronectin efficiently, suggesting that the RGD sequence in soluble fibronectin is not exposed, and that $\alpha 5$ integrin binds to the synergy site first and causes a conformational change, which exposes the RGD sequence for $\beta 1$ integrin (Huveneers et al. 2008). The idea that RGD sequence in soluble fibronectin is cryptic is also supported by studies showing that binding of the functional upstream domain (FUD) of a bacterial adhesin protein to the N-terminal portion of soluble fibronectin causes fibronectin to undergo conformational changes and expose the epitope for a monoclonal antibody that recognizes the $^{10}\text{FN3}$ module (Ensenberger et al. 2004).

Alternatively Spliced Cell-Binding Domains

$\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ integrins recognize the LDV sequence in the alternatively spliced V region of fibronectin (Guan and Hynes 1990; Mould et al. 1991; Wayner et al. 1989). It is hypothesized that LDV binds integrins at the junction between the α and β subunits similar to the way RGD binds (Humphries et al. 2006). The interaction between $\alpha 4 \beta 1$ and the V region may mediate lymphocyte adhesion under inflammatory conditions (Elices et al. 1994).

$\alpha 4 \beta 1$ and $\alpha 9 \beta 1$ integrins recognize the adjacent D (Asp) and G (Gly) residues in the C–C' loop of the EDA module (Shinde et al. 2008). EDA – $\alpha 9 \beta 1$ integrin interaction regulates fibronectin assembly in lymphatic cells and mediates lymphatic valve morphogenesis (Bazigou et al. 2009).

Effects of Fibronectin–Integrin Interactions

Integrins are heterodimeric transmembrane receptors (with α and β subunits) that interact with extracellular matrix glycoproteins, connect to the cytoskeleton inside the cell through their cytoplasmic tails, and regulate intracellular signal transduction pathways utilizing signals from extracellular ligands (Hynes 2002). Ligand–integrin interaction mediates cell adhesion, induces integrin clustering, and regulates cell shape, proliferation, differentiation, and apoptosis (Ginsberg et al. 2005). Interestingly, many of the integrin-triggered signaling pathways are similar to the growth factor-triggered signaling pathways, and most of these pathways require cells to be adherent (Hynes 2002; Schwartz and Assoian 2001). Many integrin-associated proteins, such as Src family protein tyrosine kinases, integrin-linked kinase, and protein kinase C may interact with integrins and mediate the intracellular signaling pathways (Ginsberg et al. 2005).

Fibronectin–integrin interaction may induce cytoskeleton reorganization, focal adhesion formation, actin microfilament bundle assembly, and importantly, cell-generated tension to unfold cryptic fibronectin, which is critical for fibronectin matrix assembly (Geiger et al. 2001; Hynes 1990; Mosher 1989). $\alpha 5 \beta 1$ integrin binds soluble fibronectin and supports the focal adhesion distribution, Rho activation, and fibronectin assembly (Huveneers et al. 2008). The roles of integrin in fibronectin matrix assembly are discussed in details in Sect. 2.2.4.

2.2.3.2 Collagen-Binding Domains

The collagen-binding domain of fibronectin is identified as $^6\text{FN1}$ – $^9\text{FN1}$ including the $^1\text{FN2}$ – $^2\text{FN2}$ modules (Fig. 2.1a). Fibronectin binds denatured collagen (gelatin) more effectively than native collagen (Engvall et al. 1978). Collagens denature locally at physiological temperatures and unfold their triple helices (Leikina et al. 2002), enabling fibronectin to interact with native collagen in vivo. Fibronectin–collagen interaction may mediate cell adhesion to denatured collagen, form noncovalent crosslinking of fibronectin and collagen in migratory pathways, and regulate the removal of denatured collagenous materials from blood and tissue (Mosher 1989; Pankov and Yamada 2002). Two segments of the gelatin-binding domain $^6\text{FN1}$ – $^7\text{FN1}$ (including $^1\text{FN2}$ – $^2\text{FN2}$) and $^8\text{FN1}$ – $^9\text{FN1}$ bind the same sequence of collagen $\alpha 1$ (Erat et al. 2009; Pickford et al. 2001).

2.2.3.3 Fibrin-Binding Domains

There are three fibrin-binding sites in fibronectin. The first and the major fibrin-binding site is in the N-terminal $^4\text{FN1}$ – $^5\text{FN1}$ (Williams et al. 1994). The second binding site is $^{10}\text{FN1}$ – $^{12}\text{FN1}$ close to the C-terminus. The third binding site appears following chymotrypsin digestion of fibronectin, and is immediately adjacent to the collagen-binding domain (Mosher 1989). At physiological temperatures, the

fibronectin–fibrin interaction is very weak. Covalent crosslinking of fibrin and fibronectin mediated by Factor XIII transglutaminase at a Gln residue close to the N-terminus stabilizes this interaction, helps incorporate fibronectin into the fibrin-clot, stimulates platelet thrombus growth on fibrin, and has the potential to modulate cell adhesion or migration into fibronectin–fibrin clots upon wound healing (Cho and Mosher 2006; Magnusson and Mosher 1998).

2.2.3.4 Heparin-Binding Domains

Fibronectin contains at least two heparin-binding domains that interact mainly with heparan sulfate proteoglycans. The first and strongest site localizes to ¹²FN3–¹⁴FN3 modules in the C-terminus. The crystal structure of ¹²FN3–¹⁴FN3 modules and other related studies revealed the heparin-binding site to be a group of six positively charged residues in ¹³FN3 and a minor heparin-binding site in ¹⁴FN3 (Barkalow and Schwarzbauer 1991; Ingham et al. 1990; Sharma et al. 1999). The second and weaker site is in the N-terminal ¹FN1–⁵FN1 modules. Fibronectin and heparin interact with high affinity, with at least two sets of affinities with $K_d = 10^{-7}$ to 4×10^{-9} M (Hynes 1990; Mosher 1989; Yamada et al. 1980). Other novel heparin-binding domains have been identified in ⁵FN3 module and in the alternatively spliced V region (Mostafavi-Pour et al. 2001; Moyano et al. 1999).

Heparin-binding domains may cooperate with cell-binding domain of fibronectin and potentiate cell adhesion, cell spreading, and formation of actin microfilament bundles on fibronectin for certain cell types (Beyth and Culp 1984; Izzard et al. 1986; Lark et al. 1985; Laterra et al. 1983a; Laterra et al. 1983b; Woods et al. 1986).

2.2.3.5 Bacteria-Binding Domains

Besides heparin and fibrin, the N-terminal ¹FN1–⁵FN1 can bind several types of bacteria, such as *Staphylococcus aureus* or *Streptococcus pyogenes* (Mosher 1989). Recently, much attention has been paid to the bacterial fibronectin-binding proteins (FnBPs) that mediate cell adhesion and induce entry of bacteria into nonphagocytic host cells using fibronectin (Schwarz-Linek et al. 2004). Crystal and NMR studies revealed that the FnBPs are disordered in their unbound state and upon interactions with fibronectin become ordered through an unusual and distinctive tandem β -zipper mechanism (Bingham et al. 2008) (Fig. 2.3).

2.2.4 Fibronectin Matrix Assembly

Fibronectin is important for many activities including cell migration and tissue morphogenesis (Dzamba et al. 2009; Zhou et al. 2008). These activities require

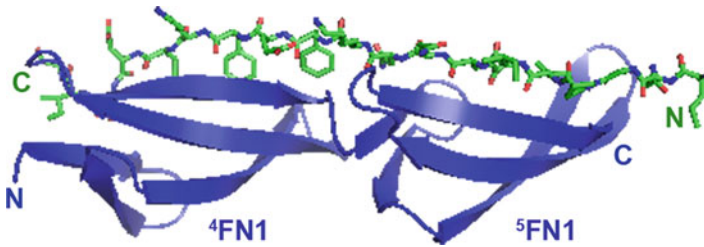


Fig. 2.3 *FnBP-1 in complex with ⁴FN1–⁵FN1 modules.* Ribbon and stick structure of ⁴FN1–⁵FN1/FnBP-1 from PDB: 2RKY (Bingham et al. 2008). Fibronectin modules are shown as ribbon in blue, and FnBP-1 forms a fourth β -strand in the major β -sheet and is shown in sticks with carbon, nitrogen, and oxygen atoms shown in green, blue, and red, respectively

fibronectin to be assembled into fibronectin fibrils, which are one of the earliest components of extracellular matrix, and provide scaffolding for deposition of the fibronectin-interacting proteins such as collagen and heparan sulfate proteoglycans in the extracellular matrix (Hynes 2009). Inhibition of fibronectin fibril formation causes delay in embryonic development (Darribere et al. 1990). Unlike assembly of collagen or laminin, fibronectin fibrillogenesis does not occur spontaneously at physiological salt concentrations and pH. It requires the presence of assembly-competent cells. The rules for fibronectin assembly seems to be the same for plasma fibronectin and cellular fibronectins (Bae et al. 2004).

2.2.4.1 Steps of Fibronectin Matrix Assembly

Soluble compact fibronectin needs to be assembled to its fibrillar matrix form in a cell-mediated, stepwise manner. Fibronectin assembly is initiated by binding of soluble fibronectin to cell surface receptors that induce conformational changes that expose cryptic binding sites in bound fibronectin. These changes facilitate fibronectin–fibronectin interactions, forming fibronectin fibrils, fibronectin fibril elongation through cell-generated tension mediated by integrins, and the formation of an insoluble fibrillar network (Fig. 2.4).

One hypothesis is that fibronectin assembly begins by interactions of the fibronectin cell-binding domain (RGD motif in ¹⁰FN3) with cell surface integrin receptors (Mao and Schwarzbauer 2005). Dimeric fibronectin induces integrin clustering by binding two integrins with its two cell-binding domains. Clustered integrins become activated, cause actin filament rearrangement, facilitate the extension of fibronectin that exposes cryptic binding sites, enable interactions of the N-terminal 70K region (¹FN1–⁹FN1, termed 70K) with other parts of fibronectin, and cause irreversible association of fibronectin to a fibrillar matrix. However, Coussen et al. found that neither monomeric nor dimeric ⁷FN3–¹⁰FN3 binds integrins stably; a trimer is required (Coussen et al. 2002), suggesting that an interacting fibronectin dimer is not sufficient to cause clustering of integrins. An alternative hypothesis is that fibronectin assembly is initiated by interaction between the N-terminal 70-kDa

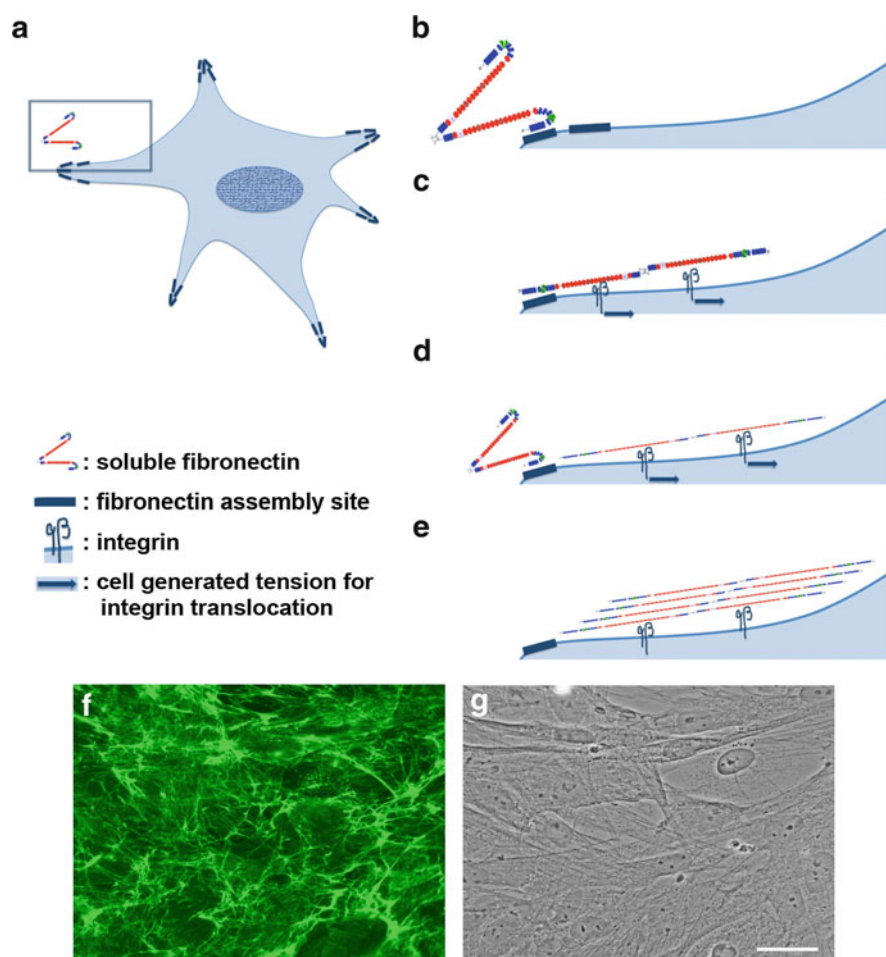


Fig. 2.4 *Hypothetical model of fibronectin assembly.* (a) Display of fibronectin assembly sites (dark blue strips at the focal adhesions) on the cell surface is controlled by the adherent substrates to which cells are attached. (b–e) show the enlarged boxed area of (a). (b) Soluble fibronectin dimer binds to linearly arrayed fibronectin assembly sites through the N-terminal 70-kDa region of fibronectin (70 K). (c) The binding of 70 K to the cell surface fibronectin assembly receptors induces unfolding of fibronectin, which exposes the RGD sequence in $^{10}\text{FN3}$. (d) The RGD-integrin (integrins are shown as “ $\alpha\beta$ ” on the cell surface) interaction activates Rho, and stretches fibronectin through tension generated from integrins and cytoskeleton contractility. Besides causing elongation of fibronectin, translocation of integrins toward the center of the cells also frees the peripheral fibronectin assembly sites for the second soluble fibronectin, and more soluble fibronectin follows. (e) Such elongation of fibronectin exposes more cryptic fibronectin–fibronectin interacting sites, leading to the formation of insoluble fibronectin fibrils through fibronectin–fibronectin interactions. (f–g) show immunofluorescence staining of fibronectin matrix. AH1F human foreskin fibroblasts were incubated in serum-containing medium for 24 h, and stained for their assembled fibronectin fibrils with an anti-human fibronectin monoclonal antibody followed by FITC-conjugated secondary antibody; an extensive network of fibrils is seen (f). Cells are shown by phase microscopy (g). Scale bar = 20 μm

region (¹FN1–⁹FN1, termed 70K) and its cell surface receptors. 70K is able to bind to fibronectin assembly sites on the cell surface without the presence of intact fibronectin (Tomasini-Johansson et al. 2006) and inhibit assembly of intact fibronectin (McKeown-Longo and Mosher 1985). In the alternative hypothesis, binding of the 70K region to cell surface receptors unfolds fibronectin, which exposes the integrin-binding site RGD to interact with cell surface integrins followed by elongation of bound fibronectin, exposing cryptic fibronectin–fibronectin interaction sites forming fibronectin fibrils.

2.2.4.2 Essential Domains for Fibronectin Matrix Assembly

The fibronectin assembly initiation site located in the N-terminal 70-kDa region (70K) is essential for fibronectin matrix assembly, especially ¹FN1–⁵FN1. A recombinant fibronectin construct including ¹FN1–⁵FN1 followed by ⁸FN3 to the C-terminus undergoes fibrillogenesis whereas removal of ⁵FN1 module from the same construct caused loss of fibrillogenesis ability (Schwarzbauer 1991). The five FN1 modules, ¹FN1–⁵FN1, likely work as a functional unit in interacting with other proteins. Removal of any of the five modules or mutation of conserved Tyr residues in individual modules results in decreased affinity (Magnusson and Mosher 1998; Sottile et al. 1991). 70K binds to the cell surface with the same affinity and at the same binding sites as intact fibronectin, but is not assembled into insoluble matrix (McKeown-Longo and Mosher 1985; Tomasini-Johansson et al. 2006). Although 70K is not assembled into insoluble matrix, 70K blocks the binding and assembly of fibronectin efficiently (McKeown-Longo and Mosher 1985).

Controversy exists as to the exact role of the cell-binding domain, especially RGD and the synergy site PHSRN. Antibodies binding to fibronectin's cell-binding domain or a fragment containing the cell-binding domain inhibited fibronectin matrix assembly in vitro (McDonald et al. 1987). Fibronectin lacking the synergy site showed reduced matrix assembly, which could be rescued by Mn²⁺, suggesting a modulatory role of the synergy site on integrin function (Sechler et al. 1997). Mouse embryos in which the RGD sequence was replaced with inactive RGE die at embryonic day 10. However, RGE-FN is assembled in fibrils in vivo (Takahashi et al. 2007). Fibronectin lacking the RGD sequence can be assembled using $\alpha 4\beta 1$ integrins (Schwarzbauer 1991; Sechler et al. 2000). Our unpublished observation with RGE-FN and fibronectin-null cells suggest that the RGD motif is not required for initial binding of soluble fibronectin, but may mediate cell adhesion, activate cells to become assembly competent and, most importantly, mediate elongation of fibronectin (unpublished data of Xu and Mosher 2009).

Besides the 70K and the cell-binding domain, there are several other regions that are essential for fibronectin matrix assembly (Pankov and Yamada 2002). Fibronectin needs to be dimeric to be assembled. Removal of the cysteines at the C-terminus of fibronectin that form the interchain disulfide bonds generates monomeric fibronectin that does not assemble. In contrast, a recombinant fibronectin

construct lacking ¹FN3–⁷FN3, that can still dimerize, is competent for fibrillogenesis (Schwarzbauer 1991).

The ability of adherent fibronectin-null fibroblasts to assemble exogenous fibronectin is dependent on the adherent substrate: cells adherent to vitronectin could not assemble exogenous fibronectin, while cells adherent to collagen, laminin, or fibronectin are competent for fibronectin assembly (Bae et al. 2004). In identification of smaller fragments in fibronectin that account for the supportive activity, the ¹FN3 module and the C-terminal modules are found to be required for activation of adherent cells to be optimally competent for fibronectin assembly (Xu et al. 2009). The mechanism of how vitronectin suppresses or how fibronectin, collagen, or laminin supports adherent cells for fibronectin assembly is obscure. Vitronectin mainly interacts with $\alpha v \beta 3$ integrin, while collagen, laminin, or fibronectin mainly interacts with $\beta 1$ integrins. $\beta 3$ integrin recycles through an endosomal “short-loop” recycling pathway, and $\beta 1$ integrin recycles through a perinuclear “long-loop” recycling pathway (White et al. 2007). It is found that the recycling of $\alpha v \beta 3$ integrin may inhibit the return of internalized $\alpha 5 \beta 1$ integrin back to the plasma membrane (White et al. 2007). Therefore, we hypothesize that for cells adherent to vitronectin, $\alpha v \beta 3$ integrin recycles rapidly and inhibits the recycling of $\alpha 5 \beta 1$ integrin, which is important for fibronectin assembly.

2.2.4.3 Role of Integrins and Cytoskeletal Contractility in Fibronectin Assembly

$\alpha 5 \beta 1$ integrins are widespread. Monoclonal antibodies to $\alpha 5$ or $\beta 1$ integrin subunits inhibited fibronectin assembly and 70K binding (Akiyama et al. 1989; Fogerty et al. 1990). Elevated levels of $\alpha 5 \beta 1$ integrin in Chinese hamster ovary (CHO) cells resulted in enhanced fibronectin assembly (Giancotti and Ruoslahti 1990). Recent studies found that the binding of $\alpha 5 \beta 1$ integrin by soluble fibronectin causes Rho activation and fibronectin assembly independent of syndecan-4 (Huveneers et al. 2008). Besides $\alpha 5 \beta 1$, other integrins like $\alpha 4 \beta 1$, $\alpha v \beta 3$, and $\alpha 9 \beta 1$ have been reported to be able to support fibronectin assembly (Akiyama et al. 1989; Bazigou et al. 2009; Sechler et al. 2000; Wennerberg et al. 1996; Yang and Hynes 1996), although other studies have also shown that $\alpha v \beta 3$ integrin could not bind soluble fibronectin and is not able to support fibronectin assembly in the absence of $\alpha 5 \beta 1$ integrin (Huveneers et al. 2008).

Fibronectin requires conformational changes to expose its cryptic sites for fibronectin–fibronectin interactions. Besides the conformational change caused by direct interaction between fibronectin and integrins, cell-driven integrin movement along the cell surface may stretch fibronectin and cause further exposure of cryptic self-association sites. Loss of cell contractility by blockage of Rho, myosin light chain kinase, or actin–myosin interaction inhibits fibronectin matrix formation (Halliday and Tomasek 1995; Wu et al. 1995b; Zhang et al. 1994; Zhang et al. 1997; Zhong et al. 1998). The majority of cryptic fibronectin–fibronectin interaction sites are in the FN3 modules (Geiger et al. 2001). The lack of disulfide bonds in

these modules is thought to facilitate the stretched-induced exposure of cryptic sites (Ohashi and Erickson 2005).

2.2.4.4 Future Prospects

Fibronectin is a late addition to the repertoire of molecules that mediate cell-extracellular matrix adhesion (discussed in Chap. 1). It can be thought of as an amalgam of FN3 modules with sites of cell adhesion and unique and distinctive FN1 modules that mediate assembly. However, how the amalgam works is still not known. A number of important questions remain unanswered. How do different adherent substrates differentially mediate adherent cells to assemble soluble fibronectin? What are the cell surface binding sites for the N-terminal 70-kDa region of fibronectin that initiates fibronectin assembly? How does fibronectin convert from soluble dimer to multimers? Which cryptic sites are required for fibronectin assembly? What are the requirements of integrins in fibronectin assembly? A better appreciation of such issues would better define the assembly of fibronectin and may be of considerable value to manipulate assembly of fibronectin matrix.

2.3 Laminin

2.3.1 Introduction

Laminins, which are present in worms and flies and are among the first extracellular matrix proteins produced during embryogenesis, are the major cell adhesive proteins of the basement membrane (Yurchenco and Wadsworth 2004) (see Chap. 4). Compared with fibronectin, which is found only in chordates, laminins are evolutionarily ancient and conserved, with sequence similarities with a laminin gene found in *Hydra vulgaris* (Tzu and Marinkovich 2008). Laminins bind cell surface receptors and thereby connect basement membrane with adjacent cell layers. Laminins are large (400–900 kDa) heterotrimeric glycoproteins of three different polypeptide chains: α , β , and γ (Fig. 2.5). Unlike fibronectin, which is encoded by a single gene and generates variants through alternative splicing, multiple genes encode each of the three laminin subunits, which can assemble in different combinations of laminin variants.

Laminins undergo self-polymerization and form filaments and layered sheets, which initiate basement membrane assembly. Interestingly, laminin sheets are generally mixtures of multiple laminins instead of separate networks of each laminin (Scheele et al. 2007). When laminin polymerization is inhibited, basement membrane assembly seems to be disrupted even in the presence of other major constituents such as entactin, type IV collagen, and perlecan (Li et al. 2002). Laminin binds cell surface receptors like heparin, integrins, and α -dystroglycan,

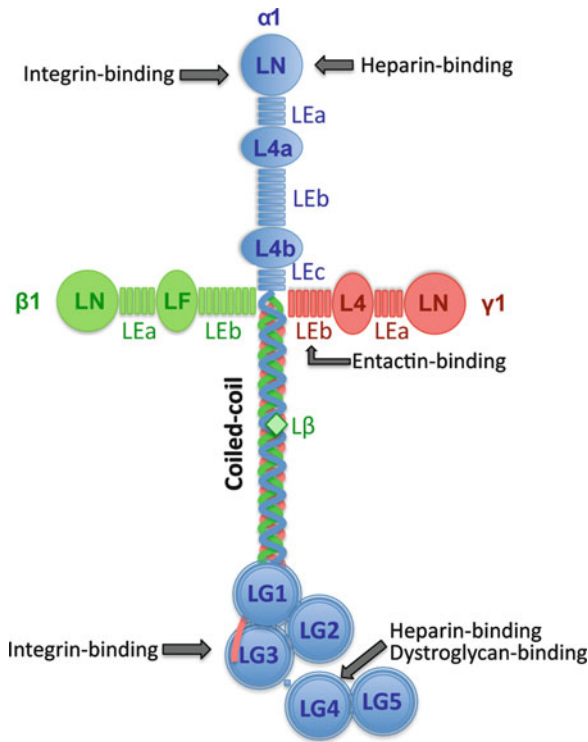


Fig. 2.5 A schematic model of the laminin-111 domain structure. Laminins are heterotrimers composed of α (blue), β (green), and γ (red) chains linked together at the coiled-coil region. The abbreviations are: LN laminin N-terminal domain, LE laminin epidermal growth factor-like domain, L4 laminin 4 domain, LF laminin four domain, LG laminin globular domain. Binding sites for heparin, dystroglycan, integrin, and entactin are indicated in the figure

which make laminin the central adhesive protein of basement membranes. Laminins mediate cell adhesion (Nomizu et al. 1998), proliferation (Kubota et al. 1992), migration (Colucci et al. 1996), and differentiation (Rozzo et al. 1993) through interaction with cell surface receptors and also play a role in neurite outgrowth (Weeks et al. 1990; Weeks et al. 1991), metastasis (Colognato and Yurchenco 2000; Malinda and Kleinman 1996), and angiogenesis (Kibbey et al. 1992). The roles of different laminins in development and disease was reviewed recently by Scheele et al. (2007), and in Chap. 4.

2.3.2 Laminin-Interacting Proteins

Laminins interact with other laminins via their N-terminal globular LN domains to self-polymerize and initiate basement membrane assembly. There are also

many protein-binding sites on laminins for extracellular matrix proteins, such as entactin (or nidogen), and for cells surface receptors, such as syndecans, integrins, and α -dystroglycan. Interestingly, most of the noncellular extracellular matrix protein-binding sites are in the short arms of the three chains, whereas most of the cell surface receptor-binding sites are in the N- and C- terminus of laminin α chains, especially in the LG domain (Timpl et al. 2000).

A major class of laminin receptor for linking cells with the basement membrane is the integrins. Laminin–integrin interaction activates a series of intracellular signaling pathways involving focal adhesion kinases (FAK), small rho GTPases, mitogen-activated protein kinases (MAPK), phosphatases, and cytoskeleton components, and therefore mediates cell adhesion, migration, proliferation, differentiation, and survival (Belkin and Stepp 2000; Givant-Horwitz et al. 2005; Gonzales et al. 1999; Hintermann and Quaranta 2004; Watt 2002).

Of the 24 different known integrin heterodimers, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 7\beta 1$, $\alpha 9\beta 1$, and $\alpha v\beta 3$ integrins have been reported to bind laminins (Nishiuchi et al. 2006; Patarroyo et al. 2002). As stated above, integrins mostly recognize laminins through the C-terminal globular LG domains of the α chains, with some integrin-binding activity at the N-terminus of α chains. The β and γ chains can be recognized by integrins as well (Patarroyo et al. 2002). Therefore, unlike the RGD and LDV sequences that define major and minor integrin-binding sites for fibronectin, the integrin-binding sites in laminins vary (Patarroyo et al. 2002). Studies of the major integrin-binding site in laminin-511 showed that deletion of the LG3 domain caused loss of its integrin-binding abilities, suggesting LG3 domain is required for integrin binding (Ido et al. 2004) (see Chap. 4 for laminin nomenclature and structure). However, recombinant LG1-3 domains do not bind integrin (Ido et al. 2006). Further studies by the same group found that Glu-1607 of the $\gamma 1$ chain and the homologous Glu residue of the $\gamma 2$ chain are critical for integrin binding, although Glu-1607 is not directly involved in integrin binding (Ido et al. 2007). Surprisingly, the $\gamma 3$ chain lacks such a Glu residue, and laminin-113 or laminin-213 is not able to bind integrins (Ido et al. 2008). When the C-terminal four residues of the $\gamma 1$ chain, including the conserved Glu residue, were swapped to the $\gamma 3$ chain, the chimeric laminin-213 regained its integrin-binding activity (Ido et al. 2008). The above results suggest that integrins bind laminin through a combination of the C-terminal conserved Glu residue of the γ chain and the LG3 domain of the α chain, although the exact binding pattern is not known. Integrins may either bind to the LG3 domain and use the Glu residue of the γ chain as an auxiliary site, or bind to a cryptic integrin-binding site in the LG3 domain exposed only upon the interaction between the LG3 domain of the α chain and the Glu residue of the γ chain (Fig. 2.5).

In addition to integrins, laminins also interact with collagen, sulfatides, heparan sulfate proteoglycans, 67-kDa laminin receptor, and α -dystroglycan (Givant-Horwitz et al. 2005; Miner and Yurchenco 2004). The LG4 domain contains a heparin-binding site that is critical for basement membrane assembly (Li et al. 2002). Other binding sites include a single entactin (or nidogen) binding site, which locates to a loop of a LEb3 domain of the $\gamma 1$ chain (Stetefeld et al. 1996). Such interaction between laminin and entactin (or nidogen) serve to bridge laminin with the collagen IV

network and has significant developmental importance (Mayer et al. 1998; Yurchenco and Schittny 1990).

2.4 Other Adhesive Glycoproteins

2.4.1 Vitronectin

Vitronectin is a 75-kDa glycoprotein present in blood plasma at a concentration of 200–400 $\mu\text{g/ml}$ (2.5–5.0 μM). It is also present in other body fluids such as amniotic fluid and urine, and in the extracellular matrix of many tissues (Preissner 1991; Tomasini and Mosher 1991). Vitronectin was independently studied under the names “serum spreading factor,” “epibolin,” and “S protein (site-specific protein)” in the late 1970s and early 1980s until investigators realized their findings relate to the same protein, vitronectin, named for its ability to bind glass. Human vitronectin is a protein of 459 amino acids mainly synthesized in the liver (Seiffert et al. 1994). In human blood, it exists in two forms: one is a single chain 75-kDa form, and the other is a two-chain form cleaved after Arg³⁷⁹ generating 65 and 10-kDa chains connected by a disulfide bridge (Cys²⁷⁴–Cys⁴⁵³) (Schvartz et al. 1999).

Vitronectin has many important protein-binding domains (Fig. 2.6). A somatomedin B domain is located at the N-terminus (amino acids 1–44) and binds plasminogen activator inhibitor-1 (Zhou et al. 2003) and interacts with the urokinase receptor (Wei et al. 1994). Immediately following the somatomedin B domain is an RGD cell adhesion sequence (residues 45–47), which is the major integrin-binding site in the protein. Adjacent to the RGD is a binding domain (amino acids 53–64) for thrombin–antithrombin complex and collagen (Schvartz et al. 1999). The core of vitronectin (residues 132–459) is homologous to hemopexin. At the C terminus, there are a plasminogen-binding site (residues 332–348) (Kost et al. 1992), two heparin-binding sites (residues 347–352 and 354–362) (Cardin and Weintraub 1989), and another plasminogen activator inhibitor-1 (PAI-1) binding site (residues

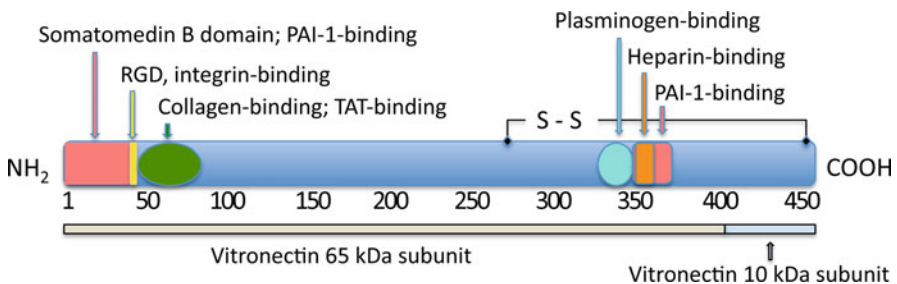


Fig. 2.6 *Protein-binding domains of vitronectin.* Vitronectin’s major ligand-binding sites are indicated, with the major integrin-binding site RGD located in residues 45–47. The disulfide bond connects the 65 and 10 kDa subunits of the cleaved form of vitronectin. PAI-1, plasminogen activator inhibitor-1; TAT, thrombin-antithrombin

348–370) (Gechtman et al. 1997). Vitronectin assumes different conformational states upon binding to ligands such as thrombin–antithrombin complex (Tomasini and Mosher 1991).

Vitronectin interacts with the extracellular matrix through its collagen- and heparin-binding domains, and with cells through its RGD integrin-binding sequence. Integrins α IIb β 3, α v β 1, α v β 3, α v β 5, α v β 8, and α 8 β 1 recognize the RGD motif of vitronectin (Brooks et al. 1994; Marshall et al. 1995; Nishimura et al. 1994; Schnapp et al. 1995; Smith et al. 1990; Thiagarajan and Kelly 1988). α 5 β 1, the major integrin receptor for fibronectin, does not recognize the RGD of vitronectin. Vitronectin–integrin interaction activates intracellular signaling pathways, induces protein phosphorylation, activates MAP kinase pathways, and mediates cell adhesion, spreading, migration, cell growth, differentiation, proliferation, and apoptosis (Felding-Habermann and Cheresch 1993; Meredith et al. 1996; Savill et al. 1990; Schwartz et al. 1999).

Vitronectin functions in wound healing, viral infection, and tumor growth and metastasis (Felding-Habermann and Cheresch 1993; Schwartz et al. 1999). Interestingly, vitronectin knockout mice developed normally with no major defects (Zheng et al. 1995), suggesting either vitronectin is dispensable or other molecule might play a rescue role in the absence of vitronectin.

2.4.2 *Thrombospondins*

Thrombospondins are a family of structurally related multifunctional, multimodular calcium-binding extracellular matrix glycoproteins encoded by separate genes. Five thrombospondins have been identified so far and can be divided into two groups: group A with thrombospondin-1 and -2 forming homotrimers, and group B with thrombospondin-3, -4, and -5 (also known as cartilage oligomeric matrix protein) forming homopentamers (Lawler 2000) (see Chap. 11). A single thrombospondin gene is present in *Drosophila* (Adams et al. 2003).

Thrombospondins have been shown to bind to cells, platelets, calcium, and various substances such as heparin, integrins, fibronectin, collagen, laminin, fibrinogen, plasminogen, osteonectin, and transforming growth factor- β ; and are important for cell adhesion and spreading, platelet aggregation, angiogenesis, neurite outgrowth, and apoptosis (Adams 1997; Esemuede et al. 2004; Frazier 1991; Mosher 1990). Various functions of thrombospondins have been mapped to different structural domains. The N-terminal domain has a high affinity heparin-binding site with roles in platelet aggregation and endocytosis of thrombospondin-1. Besides various cell-binding sites in type I repeats and the C-terminal domain of thrombospondins, there is a RGD sequence in the type III calcium-binding repeats of thrombospondin-1, -2, and -5. The RGD sequence is not conserved in all thrombospondins as it exists in thrombospondin-4 and -5 of some species but is not found in any species of thrombospondin-3. The RGD cell-adhesive motif, which is found in repeat 12 of TSP-1 and TSP-2, makes these proteins potential

ligands for $\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha 5\beta 1$, and other RGD-recognizing integrins. Main-chain and side-chain coordination of calcium by RGD, however, forces it into a conformation that would not be expected to interact with integrins (Carlson et al. 2005; Kvasnakul et al. 2004). Cell adhesion and biochemical experiments suggest that the sequence becomes active at low calcium concentrations (Chen et al. 1994; Kvasnakul et al. 2004; Lawler and Hynes 1989; Lawler et al. 1988) or after disulfide reduction (Sun et al. 1992). Thus, this may be an example of an RGD sequence that is conditionally active.

Thrombospondin-1 can inhibit endothelial cell proliferation and migration, inhibit neovascularization, and promote growth and migration of smooth muscle cells and fibroblasts (Bagavandoss and Wilks 1990; Esemuede et al. 2004; Majack et al. 1988; Vogel et al. 1993). The medical focus of thrombospondin is on the role of thrombospondin in angiogenesis and tumor therapy.

2.4.3 *Tenascins*

Tenascins are a family of extracellular matrix glycoproteins including tenascin-C, tenascin-R, tenascin-W, tenascin-X, and tenascin-Y (Jones and Jones 2000). Tenascin-C was the first tenascin identified and is mainly synthesized by the nervous system and connective tissues. Tenascin-R is found in the nervous system. Tenascin-X and tenascin-Y are found primarily in muscle connective tissues. Tenascin-W is found in kidney and developing bone with a KGD sequence that interacts with integrins (Meloty-Kapella et al. 2008). The basic structure of tenascins is variable numbers of epidermal growth factor-like repeats followed by alternatively spliced fibronectin type III modules and a fibrinogen-like globular C-terminal domain (see Chap. 11).

Like thrombospondin-1, tenascin-C contains an RGD motif and is recognized by diverse integrins, yet is classified as an antiadhesive or adhesion-modulatory protein (Orend and Chiquet-Ehrismann 2000). $\alpha 8\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 6$ integrins all bind to the RGD motif in the third fibronectin type III repeat of tenascin-C, and $\alpha 9\beta 1$ integrin binds to the same module but to a different motif: the IDG motif in sequence EIDGIELT (Joshi et al. 1993; Prieto et al. 1993; Schnapp et al. 1995; Sriramarao et al. 1993; Yokosaki et al. 1998). Similar to $\alpha 9\beta 1$, $\alpha 7\beta 1$ integrin interacts with a VFDNFVLK sequence in the alternately spliced fibronectin type-III repeat D, which corresponds to the EIDGIELT sequence for $\alpha 9\beta 1$ integrin, and both $\alpha 7\beta 1$ and $\alpha 9\beta 1$ integrins promote neurite outgrowth (Andrews et al. 2009; Mercado et al. 2004). Human umbilical vein endothelial cells adhere to tenascin-C and partially spread through $\alpha 2\beta 1$ and $\alpha v\beta 3$ integrins (Sriramarao et al. 1993).

Cell adhesion to tenascin is weak, with adherent cells being elongated instead of flattened. Adhesion usually does not result in a rearranged actin cytoskeleton as is the case with cells adherent to fibronectin (Lotz et al. 1989; Sriramarao et al. 1993). Tenascin-C causes cells adherent to fibronectin to detach through direct interaction of tenascin-C with the 13 FN3 module of FN, which inhibit the binding of syndecan-4

to fibronectin followed by suppression of focal adhesion kinase and RhoA activity (Huang et al. 2001; Midwood and Schwarzbauer 2002). The metalloprotease meprin cleaves human tenascin-C at the seventh fibronectin type III repeats and destroys the antiadhesive ability of tenascin-C by removing the C-terminal anti-adhesion domain (Ambort et al. 2010).

2.4.4 Entactins (or Nidogens)

Entactins, also known as nidogens, are ubiquitous basement membrane glycoproteins (Timpl 1989). Two entactins expressed by distinct genes have been identified in vertebrates, named entactin-1 (~150 kDa) and entactin-2 (~200 kDa) (or nidogen-1 and nidogen-2) (Kohfeldt et al. 1998). Each isoform contains three globular domains with two in the N-terminus (named G1 and G2) and the third in the C-terminus (G3). A rod-like connecting domain composed of cysteine-rich epidermal growth factor-like repeats, which include the RGD integrin-binding sequence and a thyroglobulin-like repeat, connects the N- and C-terminal globules (see Chap. 4).

Entactin-1 binds strongly to both the laminin $\gamma 1$ chain through globular domain G3 and to collagen IV through G2 (Fox et al. 1991; Poschl et al. 1996; Reinhardt et al. 1993), and serves as a link between self-assembled laminin and collagen IV to stabilize basement membrane (Timpl and Brown 1996) and integrate other extracellular matrix proteins. Entactin-1 also binds fibronectin, perlecan, and fibulins through its G2 and G3 domains (Hsieh et al. 1994; Kvensakul et al. 2001; Reinhardt et al. 1993; Sasaki et al. 1995).

The RGD integrin-binding sequence localizes to the second epidermal growth factor-like repeat in the rod-like domain. Entactin-1 mediates cell adhesion through $\alpha v \beta 3$ integrin recognizing the RGD sequence and $\alpha 3 \beta 1$ integrin recognizing a cysteine-rich epidermal growth factor repeat in the G2 globular domain (Dong et al. 1995; Gresham et al. 1996; Wu et al. 1995a; Yi et al. 1998). Mouse entactin-2 also contains a RGD sequence, but the RGD is changed to YGD in human entactin-2 (Kohfeldt et al. 1998). Mouse entactin-2 is found to mediate cell adhesion mainly through $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$ integrins from antibody inhibition studies, although the GRGDS peptide only showed low inhibition suggesting that the RGD sequence in the mouse entactin-2 is not the major integrin-binding site (Salmivirta et al. 2002). While human entactin-2 can promote cell adhesion of many different cell lines, the receptor for cell adhesion has not been identified (Kohfeldt et al. 1998).

2.4.5 Nephronectin

Nephronectin is an extracellular matrix glycoprotein identified as a novel ligand for $\alpha 8 \beta 1$ integrins, an interaction that is essential for kidney development as demonstrated in mice lacking $\alpha 8 \beta 1$ integrin or nephronectin (Brandenberger et al. 2001).

Nephronectin is 70–90 kDa, with five epidermal growth factor like-repeats (residues 57–250), an RGD-containing linker domain (residues 382–384), and a C-terminal domain with sequence homology to meprin-A5 protein-receptor protein-tyrosine phosphatase μ , named the MAM domain (residues 417–561) (Brandenberger et al. 2001). Nephronectin is widely expressed in kidney, lung, brain, uterus, placenta, thyroid gland, and blood vessels (Huang and Lee 2005) with a similar distribution as $\alpha 8 \beta 1$ integrins (Brandenberger et al. 2001; Manabe et al. 2008; Wagner et al. 2003). Mice deficient in nephronectin have a similar phenotype as $\alpha 8 \beta 1$ knockout mice with kidney agenesis and hypoplasia (Linton et al. 2007; Muller et al. 1997).

To date, $\alpha 8 \beta 1$ integrin is the only identified receptor for nephronectin. Nephronectin interacts with $\alpha 8 \beta 1$ integrin through its RGD sequence in the linker domain and a synergetic LFEIFEIER sequence on the C-terminal side of the RGD motif (Sato et al. 2009). A synthetic peptide containing both RGD and LFEIFEIER sequence binds $\alpha 8 \beta 1$ integrin $\sim 2,000$ fold better than a peptide with only the RGD motif (Sato et al. 2009). The high affinity binding of nephronectin to $\alpha 8 \beta 1$ integrin partly answers why other $\alpha 8 \beta 1$ ligands with lower affinities such as fibronectin, vitronectin, or tenascin-C are not able to compensate for the deficiency of nephronectin in kidney development.

2.4.6 Fibrinogen

The interaction of the C-terminal tail of the γ -chain of fibrinogen with $\alpha \text{IIb} \beta 3$ integrins on platelets has been subjected to extensive study because of its importance in platelet aggregation and thrombus formation. These studies have revealed a distinctive recognition motif (Springer et al. 2008). The sequence of the tail is ...GAKQAGDV in human. By crystallography, this sequence is unstructured in fibrinogen. Crystal structures of γC peptides bound to $\alpha \text{IIb} \beta 3$ revealed that the peptide binds over an extended region with interaction of carboxyl groups of the penultimate aspartate and the C-terminal valine with metals in the integrin.

2.5 Integrin Signaling Pathways

Integrins are heterodimeric transmembrane proteins composed of two subunits, α and β , each with a large extracellular domain, a transmembrane domain, and a cytoplasmic domain. It is clear from the above descriptions that integrins are the major protein cells used to both bind and respond to the adhesive glycoproteins, linking the extracellular matrix to the intracellular cytoskeleton with a bidirectional signaling pathway across the plasma membrane, with integrin extracellular domains interacting with extracellular matrix, and the integrin cytoplasmic domains linking to the cytoskeleton and signal transduction pathways (Harburger and Calderwood 2009).

Integrin activation is controlled by “inside-out” signals to achieve high-affinity binding between integrins and adhesive glycoproteins (Banno and Ginsberg 2008). And, in turn, the ligation of adhesive glycoproteins with integrins activates the “outside-in” signaling pathways regulating cell responses such as migration, survival, differentiation, and proliferation (Hynes 2002). Many integrins are expressed in an inactive state (Hynes 2002). The binding of the phosphotyrosine-binding (PTB) like domain of talin and kindlin to the cytoplasmic domain of integrin- β subunit triggers the “inside-out” integrin activation, likely through disruption of a connection between the cytoplasmic domains of the α and β subunits of integrin, which leads to tail separation and conformational changes of integrin’s extracellular domains, allowing the high affinity ligand-binding of integrins (Ginsberg et al. 2005). The reinforced ligation of integrins and adhesive glycoproteins triggers “outside-in” signals and induces integrin microclustering, conformational changes of integrin cytoplasmic domains, and recruitment of additional intracellular proteins to the integrin cytoplasmic domains forming a dynamic integrin “adhesome,” including focal adhesion kinase (FAK), Src-family protein tyrosine kinases (SFKs), Ras and Rho GTPases, integrin-linked kinase (ILK), paxillin, vinculin, and others (Ginsberg et al. 2005; Zaidel-Bar et al. 2007). Such dynamic multiprotein complex can be assembled or disassembled by altering the associated proteins through integrin phosphorylation, competitor binding, or mechanical stresses, and therefore mediate cellular responses such as adhesion disassembly and cell migration (Harburger and Calderwood 2009).

2.6 Concluding Remarks

Adhesive glycoproteins have multiple cell receptor binding sites (Table 2.1) that interact with different integrins and regulate various cell functions, including cell adhesion, migration, differentiation, growth, neurite outgrowth, apoptosis, and tumor metastases. For example, fibronectin has the RGD in 10 FN3, LDV in the V region, IDAPS in 14 FN3, KLDAPT in 5 FN3, and probably more sites that remain to be discovered. These different integrin-binding sites interact with their own sets of integrins. Thus, glycoproteins may use different integrin-binding sites to bind different cells, and cells may use different integrins to adhere to different glycoproteins. For example, fibronectin uses the EDA module to bind $\alpha 9\beta 1$ integrin of endothelial cells of the lymphatic valve, and uses RGD in 10 FN3 module to bind $\alpha 5\beta 1$ integrin of fibroblasts, while fibroblasts use $\alpha 6\beta 1$ instead of $\alpha 5\beta 1$ to bind laminins.

There are several different ways of binding integrins. The major integrin-binding sequence is the RGD sequence, which is first discovered in fibronectin and found in more than 100 other proteins, including laminin, vitronectin, thrombospondins, tenascins, collagen, entactins, and nephronectin. Other similar sequences include LDV, iso-DGR, IDAPS, and KLDAPT in fibronectin; IGD and VFDNFVLK in tenascin-C; and EGD in entactins. All of those sequences include a major residue, aspartate (D), to bind integrins. Some RGD sequences are accompanied by a synergy

Table 2.1 Summary of glycoprotein–integrin interactions

Glycoprotein	Integrin-recognition sites	Integrins
Fibronectin	RGD in ¹⁰ FN3	$\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu\beta 1$, $\alpha \text{IIb}\beta 3$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, $\alpha \nu\beta 6$
	LDV in V region	$\alpha 4\beta 1$, $\alpha 4\beta 7$
	^A FN3 (EDA)	$\alpha 4\beta 1$, $\alpha 9\beta 1$
	IDAPS in ¹⁴ FN3	$\alpha 4\beta 1$
	KLDAPT in ⁵ FN3	$\alpha 4\beta 1$, $\alpha 4\beta 7$
	³ FN3	$\alpha \beta 1$ (unknown α chain)
Laminin	Iso-DGR (spontaneously converted from NGR in ⁵ FN1 or ⁷ FN1)	$\alpha \nu\beta 3$
	Combination of C-terminal conserved Glu residue of the γ subunit and the LG3 domain of the α subunit	$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 7\beta 1$, $\alpha 9\beta 1$, $\alpha \nu\beta 3$
Vitronectin	RGD	$\alpha \text{IIb}\beta 3$, $\alpha \nu\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, $\alpha \nu\beta 8$, $\alpha 8\beta 1$
TSP-1	RGD	$\alpha \nu\beta 3$, $\alpha \text{IIb}\beta 3$, $\alpha 5\beta 1$
TSP-2	RGD	$\alpha \nu\beta 3$, $\alpha \text{IIb}\beta 3$, $\alpha 5\beta 1$
Tenascin-C	RGD	$\alpha 8\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 6$
	IDG (in EIDGELT)	$\alpha 9\beta 1$
Entactin-1	VFDNFVLK	$\alpha 7\beta 1$
	RGD	$\alpha \nu\beta 3$
Nephronectin	EGF repeat in G2	$\alpha 3\beta 1$
	RGD	$\alpha 8\beta 1$
Fibrinogen	...GAKQAGDV	$\alpha \text{IIb}\beta 3$
Collagen IV	GFOGER	$\alpha 2\beta 1$
		$\alpha 1\beta 1$

site, such as the PHSRN sequence in fibronectin and LEFIFEIER in nephronectin. The synergy site supports high-affinity integrin-RGD binding and is required for the formation of tensioned $\alpha 5\beta 1$ -fibronectin bonds. An important question for the future is whether synergy sites exist more widely in integrin-interacting proteins. Other integrin–glycoprotein interactions, including a critical GFOGER motif within a triple helical collagen peptide that binds to the I domain of $\alpha 2\beta 1$ (Zhang et al. 2003), use Glu as the critical cation-coordinating residue. $\alpha 6\beta 1$ integrin binds laminin-111 through a combination of the C-terminal conserved Glu residue of the γ subunit and the LG3 domain of the α subunit. Finally, in the case of fibrinogen, the C-terminal carboxyl group is recognized by $\alpha \text{IIb}\beta 3$. These variations upon the RGD paradigm indicate that much more needs to be learned about such fine details of ligand–integrin interactions.

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