

# Chapter 13

## Fibrin Formation, Structure and Properties

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**Abstract** Fibrinogen and fibrin are essential for hemostasis and are major factors in thrombosis, wound healing, and several other biological functions and pathological conditions. The X-ray crystallographic structure of major parts of fibrin(ogen), together with computational reconstructions of missing portions and numerous biochemical and biophysical studies, have provided a wealth of data to interpret molecular mechanisms of fibrin formation, its organization, and properties. On cleavage of fibrinopeptides by thrombin, fibrinogen is converted to fibrin monomers, which interact via knobs exposed by fibrinopeptide removal in the central region, with holes always exposed at the ends of the molecules. The resulting half-staggered, double-stranded oligomers lengthen into protofibrils, which aggregate laterally to make fibers, which then branch to yield a three-dimensional network. Much is now known about the structural origins of clot mechanical properties, including changes in fiber orientation, stretching and buckling, and forced unfolding of molecular domains. Studies of congenital fibrinogen variants and post-translational modifications have increased our understanding of the structure and functions of fibrin(ogen). The fibrinolytic system, with the zymogen plasminogen binding to fibrin together with tissue-type plasminogen activator to promote activation to the active proteolytic enzyme, plasmin, results in digestion of fibrin at specific lysine residues. In spite of a great increase in our knowledge of all these interconnected processes, much about the molecular mechanisms of the biological functions of fibrin(ogen) remains unknown, including some basic aspects of clotting, fibrinolysis, and molecular origins of fibrin mechanical properties. Even less is known concerning more complex (patho)physiological implications of fibrinogen and fibrin.

**Keywords** Fibrin formation • Fibrin structure • Fibrin properties • Fibrinogen composition •  $\alpha$ -Helical coiled-coil • Blood clot • Fibrin polymerization • Clot mechanical properties • Molecular mechanisms of fibrinolysis • Modulation of clot structure

## Abbreviations

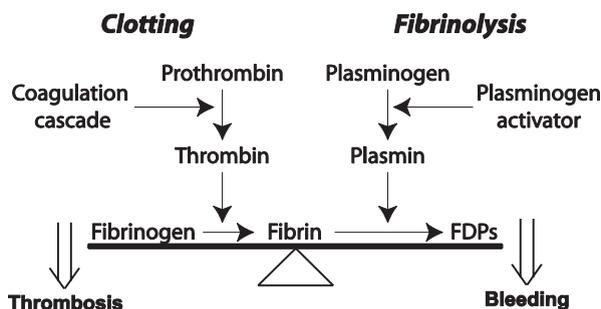
FpA and FpB	fibrinopeptides A and B
GHRP	Gly-His-Arg-Pro
GPRP	Gly-Pro-Arg-Pro
NXS or NXT	Asn-X-Ser or Asn-X-Thr

Plg	plasminogen
Pn	plasmin
t-PA	tissue-type Plg activator

### 13.1 Introduction

Fibrinogen was first classified as a fibrous protein with keratin, myosin and epidermin, based on its wide angle X-ray diffraction pattern arising from its  $\alpha$ -helical coiled-coil structure (Bailey et al. 1943). It is a 340-kDa glycoprotein, normally present in human blood plasma at a concentration of about 1.5–4 g/L, that is essential for hemostasis, wound healing, inflammation, angiogenesis, and several other biological functions. Fibrinogen is a soluble macromolecule, but forms an insoluble clot or gel on conversion to fibrin by the action of the serine protease thrombin, which is activated by a cascade of enzymatic reactions triggered by vessel wall injury, activated blood cells, or a foreign surface (Fig. 13.1). A mechanically stable clot is necessary to prevent blood loss (stopping bleeding is called hemostasis) and to promote wound healing. Fibrin clots are dissolved by the fibrinolytic system, acting in a series of enzymatic reactions with positive and negative feedback.

*In vivo*, there is a careful balance between clotting, the conversion of fibrinogen to fibrin, and fibrinolysis, the proteolytic dissolution of the clot (Fig. 13.1). Imbalance in one direction (prevalence of fibrinolysis) can lead to bleeding while the opposite imbalance (prevalence of clotting) can cause thrombosis, or formation of a clot that blocks the flow of blood through a vessel (called a thrombus). Thrombosis, often resulting from atherosclerosis or many other pathological pro-



**Fig. 13.1** Basic scheme of fibrin clot formation and fibrinolysis and the balance between these processes. The clot is formed via a cascade of enzymatic reactions that activates prothrombin to the proteolytic enzyme thrombin, which converts soluble fibrinogen to make insoluble fibrin, the process referred to as blood clotting. The fibrin clot is dissolved through fibrinolysis or cleavage by the proteolytic enzyme plasmin, resulting in fibrin degradation products (FDPs). Plasmin is formed on the fibrin surface from the zymogen plasminogen by plasminogen activators. There is a balance between clotting and fibrinolysis such that excess clotting can lead to thrombosis, while excess fibrinolysis can lead to bleeding

cesses, is the most common cause of myocardial infarction, ischemic stroke, deep vein thrombosis, and other cardiovascular diseases.

In addition to fibrin clot formation, fibrinogen is also necessary for an earlier step in hemostasis (called “primary hemostasis”), the aggregation of platelets leading to formation of a platelet “plug” at the site of vessel wall injury. The bivalent fibrinogen molecules act as bridges to link activated platelets, since the ends of rod-like fibrinogen bind with high affinity to the major adhesive receptor on platelets, the integrin  $\alpha\text{IIb}\beta_3$ . Fibrinogen also binds specifically to some other cells, but none of these cellular interactions will be discussed here, though other reviews on this topic are available (Bennett 2001; Wei et al. 2009; Collier and Shattil 2008; Collier 2011).

Fibrinogen and fibrin were last reviewed in the previous series of books dedicated entirely to Fibrous Proteins in 2005 (Weisel 2005), but many other reviews have appeared during the past 10 years that have summarized various aspects of the biology and biochemistry of fibrinogen and fibrin, though none as broad in scope (Cilia La Corte et al. 2011; Wolberg 2010, 2012; Undas and Ariens 2011; Ariens 2013; Weisel and Litvinov 2013; Weisel and Dempfle 2013; Bridge et al. 2014; Lord 2007, 2011; Falvo et al. 2010). We now know more about the process of fibrin formation, modulation of clot properties, and fibrinolysis, but perhaps the greatest explosion of new knowledge has been related to the structural origin of clot mechanical properties. Of special note for fibrous proteins, old observations of the  $\alpha$ -helix to  $\beta$ -sheet transition upon stretching of fibrin have been confirmed and a functional spring-like role for the  $\alpha$ -helical coiled-coil structure has been discovered. Although most data referenced in this review are related to human fibrinogen and fibrin, the basic principles discussed here have general relevance and are beyond species-specific peculiarities.

## 13.2 Biochemistry of Fibrinogen, the Precursor to Fibrin

### 13.2.1 Biosynthesis of Fibrinogen in Hepatocytes

Fibrinogen is the product of three closely linked genes, *FGA*, *FGB*, and *FGG*, each specifying the primary structure of one of its three polypeptide chains,  $A\alpha$ ,  $B\beta$ , and  $\gamma$ , respectively (Chung et al. 1983, 1990; Crabtree 1987). The fibrinogen genes are clustered on human chromosome 4 (Kant et al. 1985) and they translate into nascent polypeptides of pre-pro- $A\alpha$  chain (644 amino acid residues), pre-pro- $B\beta$  chain (491 residues), and pre-pro- $\gamma$  chain (437 residues). An extension of an open reading frame into an alternately spliced sixth intron gives rise to a longer  $A\alpha$  chain ( $\alpha\text{E}$ ) in 1–2 % of molecules in the adult (more in fetal blood), so that they contain an additional domain that is homologous to the C-terminal  $B\beta$  and  $\gamma$  chains, resulting in a fibrinogen molecule with a molecular mass of 420 kDa compared to 340 kDa for the major fibrinogen fraction with the shorter  $A\alpha$  chains (Fu and Grieninger 1994). The alternative splicing of a  $\gamma$  chain mRNA produces 8–15 % of plasma fibrinogen

molecules in which the  $\gamma$  chain C-terminal 400–411 dodecapeptide ( $\gamma$ A chain) is altered by adding new amino acids from 408 to 427 ( $\gamma'$  chain) (Wolfenstein and Mosesson 1981; Chung and Davie 1984; de Maat and Verschuur 2005).

Before fibrinogen is secreted from hepatocytes into blood, it needs to undergo several steps of assembly of the polypeptide chains. Following translation of each of the chains, independent translocation into the lumen of the endoplasmic reticulum, interactions of the chains with chaperone proteins that assist in the assembly and folding processes, and quality control mechanisms that distinguish properly assembled fibrinogen destined for secretion from unassembled forms that are degraded intracellularly (Redman and Xia 2001; Tamura et al. 2013). During translocation of the single polypeptides into the lumen of the endoplasmic reticulum, a signal peptide is cleaved from each chain. After full processing and assembly in the endoplasmic reticulum and Golgi organelles, there are 610, 461, and 411 amino acids in each of the common final forms of the human  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chains, respectively, remaining from the corresponding nascent pre-pro-polypeptides. In the endoplasmic reticulum there is a progression from single chains to two-chain complexes to trimeric half molecules generated by combining a  $\text{B}\beta$  chain with the  $\text{A}\alpha$ - $\gamma$  dimer or an  $\text{A}\alpha$  chain with the  $\text{B}\beta$ - $\gamma$  dimer. Next, the two trimers are joined at their N-termini to form the hexamer via disulfide bridges necessary for assembly of the two half molecules (Zhang and Redman 1996). Although the distal clusters of inter-chain disulfide bonds (named “disulfide rings”) are not necessary for assembly of the two half molecules, they are necessary for secretion. In addition to assembly and formation of disulfide bonds, before secretion fibrinogen undergoes a number of co-translational and post-translational modifications, including N-glycosylation, these are discussed below. The structure of fibrinogen can be modified even after it has been secreted into the blood, for example by limited proteolysis and glycation.

### 13.2.2 Fibrinogen Metabolism

The liver is the primary source of plasma fibrinogen, with a steady state rate of synthesis of 1.7–5 g per day and a large intracellular reserve (Takeda 1966). Three quarters of human fibrinogen is present in the plasma but it is also in platelets, lymph and interstitial fluid. The normal half-life of fibrinogen is 3–5 days, but despite the numerous studies on the distribution of iodine-labeled fibrinogen, its physiological catabolic pathway is largely unknown. Coagulation and fibrinolysis accounts for only 2–3 % of fibrinogen loss in healthy individuals (Nossel 1976). Moreover, fibrinogen turnover in the presence and absence of heparin, a potent inhibitor of blood clotting, did not reveal any differences in the half-life of fibrinogen, so intravascular fibrin formation was not found to be a quantitatively significant route in fibrinogen catabolism under physiological conditions in humans (Takeda 1966; Collen et al. 1972). Another proposed pathway of fibrinogen catabolism is proteolytic degradation by plasmin; however, in the presence of tranexamic acid, an inhibitor of fibrinolysis, the half-life of labeled fibrinogen did not change (Collen

et al. 1972). Nevertheless, proteolytic fibrin(ogen) degradation products appear to play a role in the regulation of fibrinogen turnover (Nham and Fuller 1986).

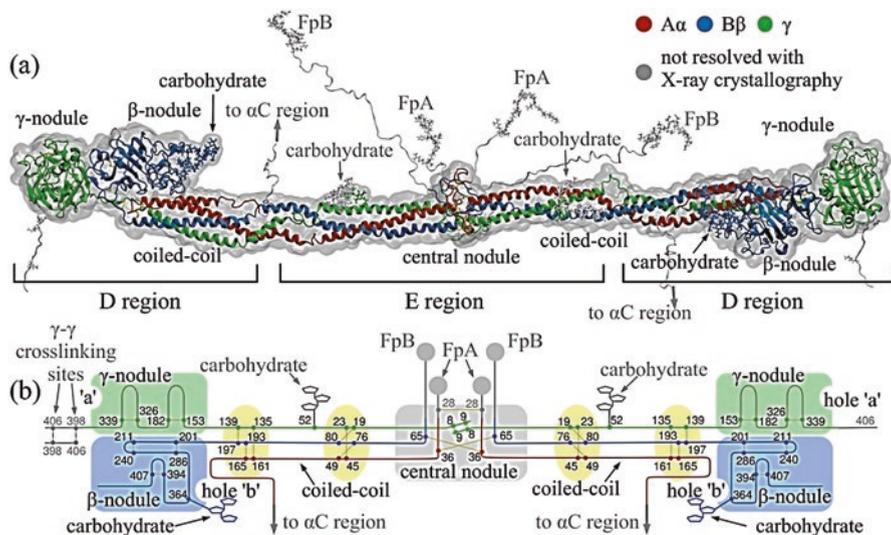
Fibrinogen is one of the acute phase proteins, which are up-regulated in response to injury and inflammation, followed by an up to ten-fold increase in its concentration in the blood (Crabtree 1987). The up-regulation is mediated largely by interleukin-6 and perhaps other pro-inflammatory mediators that trigger intracellular signaling pathways in hepatocytes and modulate gene expression via various transcription factors (Hantgan et al. 2000; Fish and Neerman-Arbez 2012).

There is intracellular fibrinogen stored in platelet  $\alpha$ -granules, but its origin is controversial, as is whether it is structurally and functionally distinct from plasma fibrinogen. A separate origin is suggested by the observation that the  $\gamma$  chain variants of fibrinogen appear to be absent in platelets (Haidaris et al. 1989), and in one patient with a heterozygous genetically modified fibrinogen, the platelets contained only normal fibrinogen (Jandrot-Perrus et al. 1979). Since platelets originate from megakaryocytes, the latter are also a possible source of fibrinogen synthesis. However, in subjects with a low fibrinogen level in blood (hypofibrinogenemia), there are also lower levels of fibrinogen in platelets, and infusion of fibrinogen results in a subsequent increase in platelet fibrinogen (Harrison et al. 1989). Also, no fibrinogen mRNA has been found in megakaryocytes (Louache et al. 1991). Therefore, it appears that platelet and megakaryocyte fibrinogen arise primarily from  $\alpha$ IIb $\beta$ 3-mediated internalization of plasma fibrinogen (Harrison et al. 1990).

Although the liver is the primary source of plasma fibrinogen, fibrinogen is also synthesized in some extra-hepatic tissues. Only fibrinogen  $\gamma$  chain gene expression has been shown *in vivo* in bone marrow, brain, and lung (Haidaris and Courtney 1990). Epithelial cells from lung and intestine secrete small amounts of fibrinogen in a polarized manner from their basolateral face (Haidaris 1997). It is possible that lung epithelium secretes fibrinogen and incorporates it into the extracellular matrix under certain pathological conditions, contributing to fibrotic lung disease, because the amount of fibrinogen expressed in lung epithelial cells is dramatically increased after treatments with dexamethasone and interleukin-6 (Lawrence and Simpson-Haidaris 2004). Synthesis of fibrinogen by cultured granulosa cells may reflect a possible function for it in ovulation (Parrott et al. 1993). The apparent *in vivo* synthesis of fibrinogen by trophoblasts (Galanakis et al. 1996) and the fact that the trophoblast basement membrane consists largely of fibrin(ogen) suggest that these cells may secrete fibrinogen into their abluminal and/or interstitial environment, but the functional significance is as yet unknown. Taken together, the normal biological relevance of the synthesis of fibrinogen in extra-hepatic tissues is unclear, but it may become important under certain pathological circumstances.

### 13.2.3 Polypeptide Chain Composition of Fibrin(ogen)

Human fibrinogen is made up of three pairs of polypeptide chains, designated A $\alpha$ , B $\beta$  and  $\gamma$ , with molecular masses of 66,500, 52,000, and 46,500 Da, respectively (Fig. 13.2). The co- and post-translational addition of N-linked carbohydrate to the



**Fig. 13.2** Fibrinogen structure. (a) The atomic resolution structure of about two-thirds of the fibrinogen molecule has been determined by X-ray crystallography (PDB Entry: 3GHG). Fibrinogen and its parts are shown with addition of portions missing from the crystal structure reconstructed computationally, namely the amino terminal ends of the  $\alpha$ A and  $\beta$ B chains with FpA and FpB in the central nodule and the beginning of the  $\alpha$ C regions. (b) Schematic diagram of the polypeptide chains of fibrinogen. The  $\alpha$ A,  $\beta$ B and  $\gamma$  chains are represented by lines with lengths proportional to the number of amino acid residues in each chain and various structural regions are labeled (Zhmuov et al. 2011, with permission from Elsevier Ltd.)

$\beta$ B and  $\gamma$  chains brings the total molecular mass to about 340 kDa. The nomenclature for the polypeptide composition of fibrinogen ( $\alpha$ A  $\beta$ B  $\gamma$ )<sub>2</sub>, arises from the designation of the small fibrinopeptides A and B (FpA and FpB) that comprise the N-terminal ends of the  $\alpha$ A and  $\beta$ B chains, respectively, and are cleaved by thrombin to yield the  $\alpha$  and  $\beta$  chains without the fibrinopeptides. No peptides are cleaved by thrombin from the  $\gamma$  chains, hence the subunit composition of monomeric fibrin is ( $\alpha$   $\beta$   $\gamma$ )<sub>2</sub> and the conversion of fibrinogen into fibrin monomer can be described as ( $\alpha$ A  $\beta$ B  $\gamma$ )<sub>2</sub>  $\rightarrow$  ( $\alpha$   $\beta$   $\gamma$ )<sub>2</sub> + 2FpA + 2FpB.

All six chains are held together by 29 disulfide bonds (Henschen and McDonagh 1986) to make two symmetrical half-molecules (Fig. 13.2). There are 8, 11, and 10 cysteine residues in the  $\alpha$ A,  $\beta$ B, and  $\gamma$  chains, respectively, and the amino termini of all six chains are held together by disulfide bonds in the central globule. Unusual Cys-Pro-X-X-Cys sequences occurring twice in each chain are arranged into “disulfide ring” structures, in which all three chains are joined together at each end of the  $\alpha$ -helical coiled-coils (Doolittle 1984). Three interchain disulfide bonds link the two halves of the molecule together, one between the two  $\alpha$ A chains and two between the two  $\gamma$  chains. A single interchain disulfide bond connects the  $\alpha$ A and  $\beta$ B chains within each half-molecule. The remainder of the  $\alpha$ A chain contains one intrachain disulfide, while the  $\beta$ B chain contains three intrachain disulfides and the  $\gamma$  chain contains two.

### 13.2.4 Overall Structure of Fibrinogen Molecules

Based on transmission electron microscopy, atomic force microscopy, and X-ray crystallographic data, the fibrinogen molecule has an elongated shape 45 nm in length and ~2–5 nm in diameter (Fig. 13.2; Hall and Slayter 1959; Fowler and Erickson 1979; Williams 1981; Weisel et al. 1985). Plasmin cleaves fibrinogen into a number of core fragments, namely fragment E comprising the central part of the molecule and two identical fragments D originating from the lateral parts of fibrinogen (Nussenzweig et al. 1961). According to the nomenclature of the corresponding proteolytic fragments, the fibrinogen molecule has two distal globular D regions and one central globular E region each containing a part of  $\alpha$ -helical coiled-coils (Medved and Weisel 2009) (Fig. 13.2). The atomic resolution structure of more than two-thirds of fibrinogen has been accomplished through X-ray crystallography (Yee et al. 1997; Spraggon et al. 1997; Brown et al. 2000; Madrazo et al. 2001), though the structure still has not been completed, since there are missing unstructured portions, which are not resolved crystallographically. These are residues 1–26, 1–57, and 1–13 at the N-termini of the  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chains, respectively, and residues 201–610, 459–461, and 395–411 at the C-termini of the  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chains, respectively. These unresolved portions have been reconstructed computationally (Zhmuurov et al. 2011) to gain a complete molecular structure of fibrinogen. Unlike other crystallographically unresolved parts of fibrinogen, the 390 residue-long C-termini of the  $\text{A}\alpha$  chains (residues 221–610), named the  $\alpha\text{C}$  regions, were visualized using transmission electron microscopy (Veklich et al. 1993) and atomic force microscopy (Protopopova et al. 2015). The  $\alpha\text{C}$  regions fold back from the distal ends of the triple coiled-coils to form a fourth strand and then extend outward via a flexible connector to relatively compact C-terminal domains that interact with the central globule of fibrinogen (Veklich et al. 1993; Litvinov et al. 2007b). Truncation of the  $\alpha\text{C}$  regions affects substantially the hydrodynamic behavior of fibrinogen (Raynal et al. 2013).

### 13.2.5 Domain Structure of Fibrinogen

Fibrinogen is organized into domains, or independently folded structural units (Weisel 2005). From the X-ray crystallographic studies of fibrinogen, the central E region has four domains and each D region comprises seven domains (Medved and Weisel 2009; Fig. 13.2). The E region is composed of two symmetrical parts, in which the C-terminal parts of the  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chains form a *coiled-coil-E domain* comprising a triple  $\alpha$ -helical structure. The N-terminal parts of two  $\gamma$  chains form an asymmetric  *$\gamma\text{N}$ -domain* domain in the center of the E region. Another domain, called the *funnel-shaped domain*, is formed on the opposite side of the E region by the parts of two  $\text{A}\alpha$  and two  $\text{B}\beta$  chains. The globular part of the E region without the

coiled-coil-E domains is often called the *central nodule*. In the lateral D region, the N-terminal parts of all the  $A\alpha$ ,  $B\beta$  and  $\gamma$  chains form a *coiled-coil-D domain* comprising a triple  $\alpha$ -helical structure. The C-terminal parts of the  $\beta$  and  $\gamma$  chains make up the  *$\beta$ -nodule* and  *$\gamma$ -nodule*, respectively. They are also named  $\beta$ - or  $\gamma$ -modules and each is made of three domains. As determined in the crystal structure of the  $\gamma$ -module these domains are named *A-domain* (N-terminal), *B-domain* (central) and *P-domain* (C-terminal; Medved and Weisel 2009).

Although the structure of the remaining fibrinogen regions not present in the crystal structures is not as well defined, it is known from NMR studies that in human fibrinogen each  $\alpha C$  region (residues  $A\alpha 221$ – $610$ ) forms two relatively compact structures. Namely, the C-terminal part (residues  $A\alpha 392$ – $610$ ) contains a distinct structure connected to the rest of the molecule via the N-terminal portion (residues  $A\alpha 221$ – $391$ ) comprising a flexible unstructured tether. As a result, the compact part is referred to as the  *$\alpha C$ -domain* and the flexible part as the  *$\alpha C$ -connector*. Notably, the two unstructured  *$B\beta N$  regions* comprising the flexible N-terminal portions of the  $B\beta$  chains contain a number of functionally important binding sites (Gorkun et al. 2006).

### 13.2.6 $\alpha$ -Helical Coiled-Coils of Fibrinogen

The central nodule and end globular parts of fibrin(ogen) are joined together by 17-nm-long triple (and partially quadruple)  $\alpha$ -helical coiled-coil connectors formed by 111 or 112 amino acid residues from each of the  $A\alpha$ ,  $B\beta$  and  $\gamma$  chains that are bordered by “disulfide rings” (Fig. 13.2). In the  $\alpha$ -helical coiled-coil, three right-handed  $\alpha$ -helices wind around each other to form a left-handed supercoil (Cohen and Parry 1990). Unlike most triple helices, each of the coiled-coils of fibrinogen has a fourth helix in the bundle containing 30 residues ( $A\alpha$ Ser166 to  $A\alpha$ Pro195) that begins at the lateral disulfide ring where the  $A\alpha$  chain makes a U-turn and stretches in the reverse direction for about  $\frac{1}{4}$  of the length of the coiled-coil connector (Spraggon et al. 1997). The coiled-coils can bend around a central hinge point located in a non-helical segment of the  $\gamma$  chain adjacent to the carbohydrate attachment site  $\gamma$ Asn52 (Marsh et al. 2013). The bent portion of the coiled-coil exposes the cleavage sites that can be hydrolyzed by plasmin and other proteases followed by degradation of fibrinogen into proteolytic fragments. It also explains the conformational flexibility of fibrinogen observed in solution and at interfaces (Kohler et al. 2015). The functional role of the coiled-coil region has been recently ascribed to the tensile deformation of fibrin fibers, namely to the ability to undergo partial unraveling and spring-like reversible extension-contraction that helps to accommodate and propagate the tensile stress along the fiber axis (Zhmurov et al. 2011). At a high extent of tensile or compressive deformation, the  $\alpha$ -helices undergo mechanical conversion into  $\beta$ -sheets (Litvinov et al. 2012; Zhmurov et al. 2012; see Sect. 13.5.5).

### 13.2.7 *Ca<sup>2+</sup>-Binding Sites in Fibrinogen*

Fibrinogen has both strong and weak binding sites for calcium ions, which are important for its functions, including fibrin polymerization, and lytic stability. Although biochemical experiments originally suggested that there were three high-affinity calcium binding sites in fibrinogen (Marguerie et al. 1977), the binding sites were identified from X-ray crystallographic structures. High-affinity binding sites (named  $\gamma 1$ ) for calcium ions are present in the  $\gamma$  chains and are associated with four coordinating amino acid residues, namely  $\gamma$ Asp318,  $\gamma$ Asp320,  $\gamma$ Gly324, and  $\gamma$ Phe322, and two strongly bound water molecules (Yee et al. 1997; Spraggon et al. 1997). Other high-affinity  $\text{Ca}^{2+}$ -binding sites (named  $\beta 1$ ) are located in the  $\beta$ -nodules in loop  $\beta 381$ – $385$ , each of which has one coordinating water molecule (Everse et al. 1998b). The dissociation constant for calcium ions in these binding sites is high enough that both types of these sites will be fully occupied in fibrinogen at physiological  $\text{Ca}^{2+}$  concentrations.

Two other  $\text{Ca}^{2+}$ -binding sites named  $\gamma 2$  and  $\beta 2$  have much lower affinities. The  $\gamma 2$  sites are located in the loops  $\gamma 294$ – $301$  (Everse et al. 1999). Impairment of these sites by mutating residues  $\gamma$ Asp298 and  $\gamma$ Asp301 caused only moderate effects on the crystal structure and functional properties of fibrinogen (Kostelansky et al. 2007). It is likely that the  $\gamma 2$  sites are formed as a result of molecular rearrangements induced by crystal packing (Kostelansky et al. 2004a, b). The low-affinity  $\text{Ca}^{2+}$ -binding sites  $\beta 2$  are formed by residues  $\text{B}\beta$ Asp261,  $\text{B}\beta$ Asp398, and  $\gamma$ Glu132 and the backbone carbonyl oxygen of  $\text{B}\beta$ Asp263 (Everse et al. 1999; Kostelansky et al. 2002, 2004a, b). The  $\beta 2$  sites anchor the  $\beta$ -nodules to the coiled-coil connector and were shown to be involved in the lateral aggregation of protofibrils (Kostelansky et al. 2004b). It has been proposed that the  $\beta 2$  sites regulate accessibility of the tissue plasminogen activator (t-PA)-binding site in fibrin(ogen) (Doolittle and Pandi 2006). There may also be some low affinity  $\text{Ca}^{2+}$ -binding sites associated with the sialic acid residues on the carbohydrate chains (Dang et al. 1989).

When calcium ions are bound to the  $\gamma$  chain high affinity sites, the  $\gamma$  chains are protected from enzymatic degradation (Ly and Godal 1973; Odrliin et al. 1996), similar to the way that the peptide Gly-Pro-Arg-Pro protects the molecule from plasmin digestion (Yamazumi and Doolittle 1992).  $\text{Ca}^{2+}$ -binding does not affect FpA cleavage by thrombin or batroxobin, but appears to be important for modulating fibrin polymerization by enhancing lateral aggregation to form thicker fibers, so that mutations affecting the high-affinity  $\text{Ca}^{2+}$ -binding site have severe functional consequences (Brennan et al. 2007). There is a conformational change in fibrin associated with FpB cleavage that is  $\text{Ca}^{2+}$ -dependent (Dyr et al. 1989; Donovan and Mihalyi 1985; Mihalyi 1988). There is also a change in affinity of  $\text{Ca}^{2+}$ -binding sites associated with FpB release and the accompanying conformational change. Gly-His-Arg-Pro, a knob 'B' mimetic peptide, binds 10-fold more strongly to fibrinogen in the presence of  $\text{Ca}^{2+}$  than in its absence, which may be related to the  $\beta 2$  site that is involved in the conformational change associated with binding of Gly-His-Arg-Pro (Everse et al. 1999).

### 13.2.8 Carbohydrate Moieties of Fibrinogen

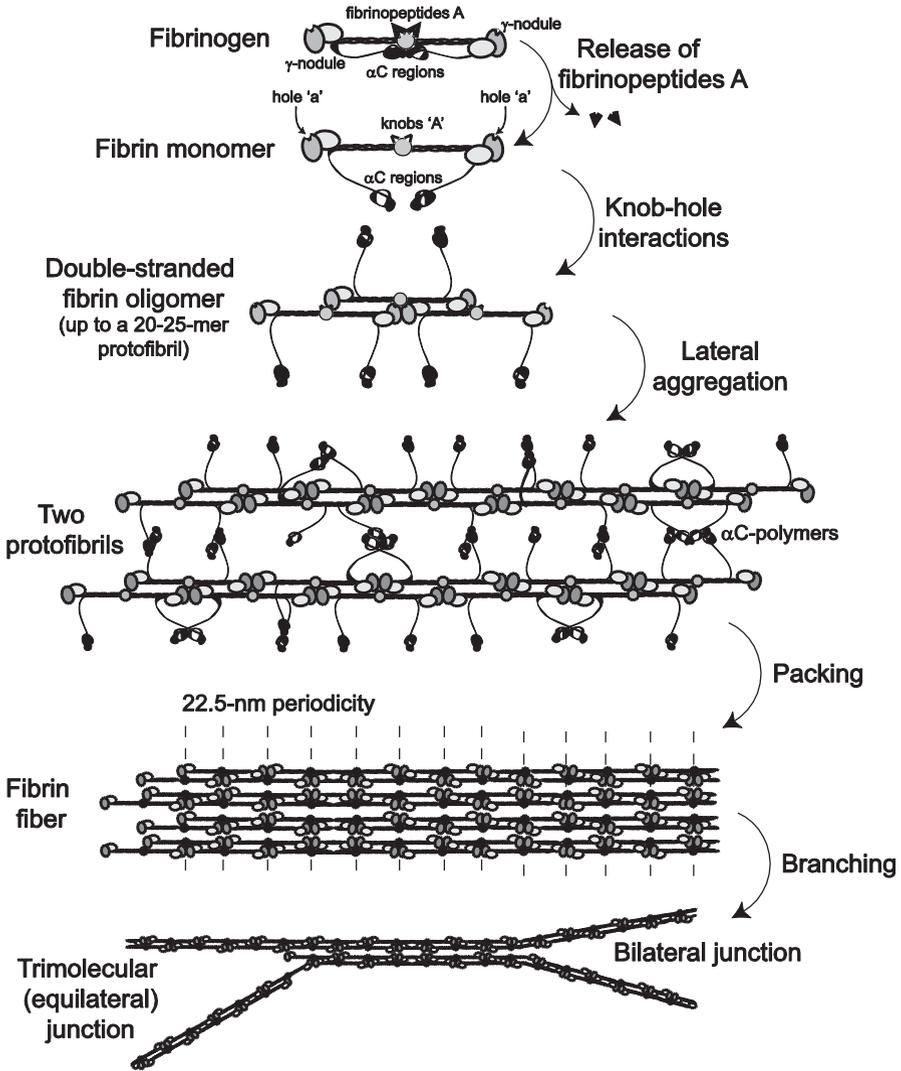
Four oligosaccharide chains are linked to each molecule of fibrinogen by way of N-glycosidic bonds: two are connected to the B $\beta$ Asn364 residues in the  $\beta$ -nodule (resolved in the crystal structure 3GHG) and the other two are connected to  $\gamma$ Asn52 in the coiled-coils (unresolved crystallographically) (Fig. 13.2). These carbohydrate attachment sites contain the classic NXS or NXT sequences that are typical of N-glycosylation. On the other hand, the A $\alpha$  chains are devoid of any carbohydrate, in spite of the presence of two NXS sequences. Variable desialylation, or removal of the terminal N-acetylneuraminic acid residue (sialyl), accounts for part of the heterogeneity of circulating fibrinogen. Fibrinogen isolated from human plasma contains equal amounts of mono- and di-sialylated carbohydrate chains but no asialo-chains (Townsend et al. 1982, 1984).

The carbohydrate on fibrinogen has striking consequences for fibrin polymerization and for clot structure. Patients with cirrhosis of the liver and some other liver diseases have fibrinogen with high levels of sialylation of their carbohydrate, resulting in fibrin networks containing thinner fibers with a higher density of branch points (Martinez et al. 1983). These results are consistent with studies using neuraminidase to remove sialic acid from the carbohydrate of normal fibrinogen, producing clots made up of thicker fibers (Dang et al. 1989). Fibrinogen synthesized in inflammatory conditions (acute phase fibrinogen) has substantially different oligosaccharide structure (Brennan 2015). Complete removal of carbohydrate has more striking effects on clot structure, resulting in clots made up of very thick fibers (Langer et al. 1988). These results suggest that both the charge and mass of the carbohydrate help to modulate the extent of lateral aggregation and that the carbohydrate moieties significantly enhance the solubility of fibrinogen. Based on computational reconstructions, it has been recently proposed that the bulky carbohydrate moieties can potentially affect the 'B-b' knob-hole interactions either by tethering the knob 'B' ( $\gamma$ Asn52) or obstructing the hole 'b' (B $\beta$ Asn364), or both (Zhurov et al. in preparation).

## 13.3 Molecular Mechanisms of the Conversion of Fibrinogen to Fibrin

### 13.3.1 General Remarks

Conversion of fibrinogen to fibrin is one of the major consequences of the enzymatic cascade of blood coagulation that is essential for stopping bleeding (hemostasis), as well as in vascular obstruction or thrombosis. It occurs in two major steps: enzymatic and non-enzymatic (Fig.13.3). At the enzymatic step, there is thrombin-catalyzed cleavage of the fibrinopeptides of fibrinogen to form the fibrin monomer. Thrombin is a highly specific serine protease upon activation of its zymogen,



**Fig. 13.3** Schematic diagram of fibrin polymerization. Fibrinopeptides in the central nodule cover knobs that are complementary to holes that are always exposed at the ends of the protein. When the fibrinopeptides are removed by thrombin, knob-hole interactions occur, giving rise to oligomers (a trimer is shown), which elongate to produce the two-stranded protofibrils made up of half-staggered molecules. The protofibrils aggregate laterally to make fibers, a process enhanced by interactions of the  $\alpha$ C regions and formation of the  $\alpha$ C-polymers. The fiber has a 22.5 nm periodicity as a result of half-staggering of 45-nm molecules. At the bottom of the diagram, branch points have been initiated by the divergence of two protofibrils (*right*) and splitting of each strand of a single protofibril (*left*) (Weisel and Litvinov 2013; Weisel and Dempfle 2013)

prothrombin, normally present in the blood. At the non-enzymatic stage, the monomeric fibrin self-assembles spontaneously to yield fibrin oligomers that lengthen to make two-stranded protofibrils. Protofibrils aggregate both laterally and longitudinally to form fibers that branch to yield a three-dimensional gelled network called a clot. Finally, the fibrin polymer is stabilized via covalent crosslinking by a plasma transglutaminase, Factor XIIIa, to form a mechanically and chemically more stable mature fibrin clot.

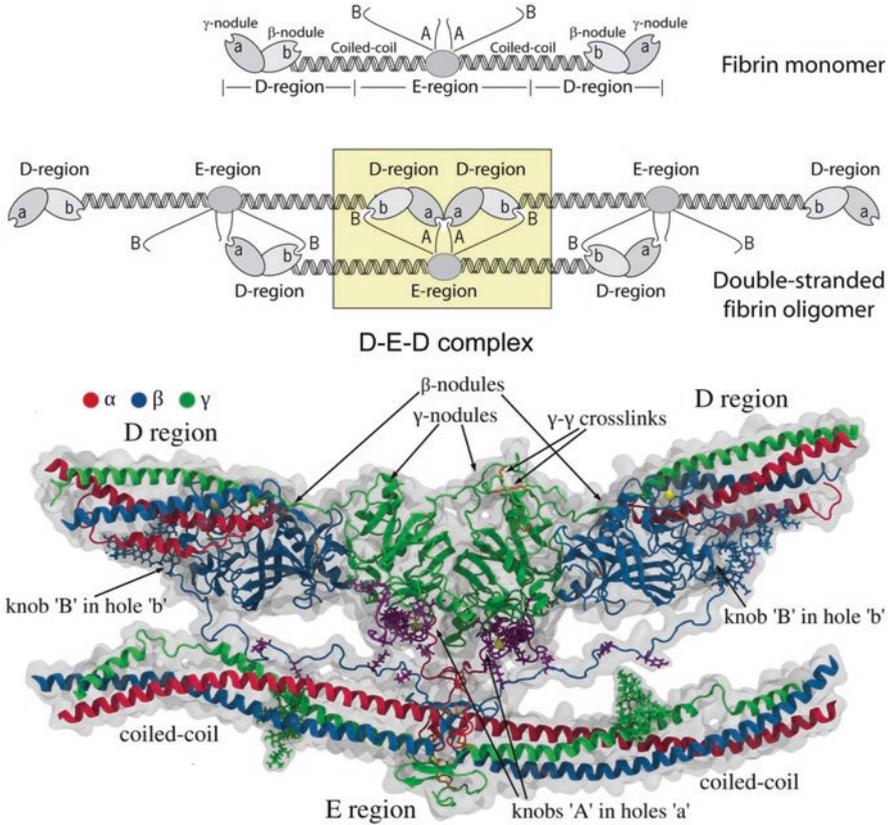
### ***13.3.2 Enzymatic Release of Fibrinopeptides from Fibrinogen***

Fibrin polymerization is triggered when thrombin cleaves FpA and FpB from the N-terminal portions of the A $\alpha$  and B $\beta$  chains of fibrinogen, respectively, producing monomeric fibrin. FpA (residues 1–16) is cleaved at the A $\alpha$ Arg16-Gly17 peptide bond, while FpB (residues 1–14) is cleaved at the B $\beta$ Arg14-Gly15 bond, albeit more slowly than FpA. With polymerization, the rate of release of FpBs increases, reaching maximum when polymerization is almost complete, indicating that they are preferentially released from fibrin polymer (Erickson and Fowler 1983; Weisel et al. 1993). In surface-attached fibrinogen, unlike in solution, FpBs are cleaved at a faster rate than FpAs, depending on fibrinogen surface density and orientation (Riedel et al. 2011), indicating that the conformation of fibrinogen determines the ability of thrombin to access and cleave FpAs and FpBs differentially. This distinction in the cleavage rate of FpA and FpB is based on the spatial restrictions of the binding of thrombin to fibrinogen, implying that the N-termini of the A $\alpha$  chain containing FpA are more accessible to the active site of thrombin (Pechik et al. 2006). When thrombin cleavage sites are mutated at positions A $\alpha$ Arg16 or B $\beta$ Arg14 (in dysfibrinogenemias or recombinant fibrinogens) the release of FpA or FpB is precluded, leading to impaired fibrin formation (Galanakis et al. 1989; Moen et al. 2003).

### ***13.3.3 ‘A-a’ Knob-Hole Interactions in Fibrin***

After cleavage of FpAs, the  $\alpha$  chains have new N-terminal sequences Gly-Pro-Arg- (GPR) named knobs ‘A’ (Medved and Weisel 2009). During fibrin polymerization, knobs ‘A’ interact with holes or pockets ‘a’ that are always open in the  $\gamma$ -nodules of the interacting fibrin molecules; the binding of knobs ‘A’ to holes ‘a’ is called the ‘A-a’ interaction (Figs. 13.3 and 13.4). The ‘A-a’ interactions have been characterized biophysically at the single-molecule level as quite strong, highly specific, and stable intermolecular associations (Litvinov et al. 2005).

Structures of fragment D (corresponding to the lateral D regions containing the  $\gamma$ -nodule) co-crystallized with the peptide GPRP (synthetic knob ‘A’ mimetic) clearly indicated that this ligand was located in hollows present in the  $\gamma$ -nodule



**Fig. 13.4** Complementary binding sites or knob-hole interactions in fibrin polymerization. *Top.* Schematic diagram of knob-hole interactions. Knobs 'A' and 'B' in the central domain of a fibrin monomer are complementary to holes 'a' and 'b' that are always exposed at the ends of the protein. When the fibrinopeptides are removed by thrombin, exposing the knobs, knob-hole interactions occur, giving rise to the trimer shown and eventually to the two-stranded protofibril made up of half-staggered molecules. *Bottom.* Atomic resolution structure of the knob-hole interactions. The  $\gamma$ - and  $\beta$ -nodules near the ends of the molecule contain the holes 'a' and 'b', respectively, that are complementary to the knobs 'A' and 'B' in the central nodule. Most of these structures were derived from X-ray crystallographic data, although the disordered and/or flexible N-terminal regions of the  $\alpha$  and  $\beta$  chains were derived from computational modeling (with permission from Elsevier Ltd.)

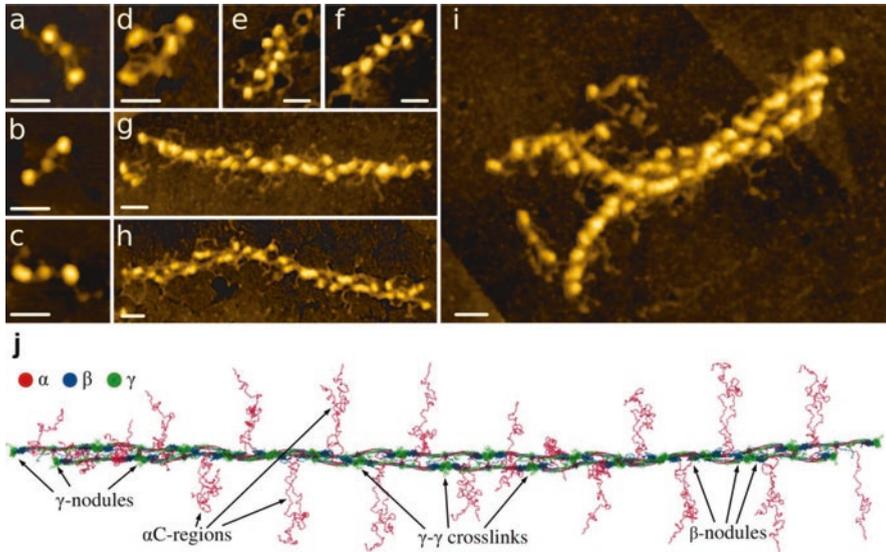
(Everse et al. 1998b; Kostelansky et al. 2002), evidence that these are holes 'a'. The hot spots of holes 'a' directly involved in the binding of the GPRP peptide were identified as residues  $\gamma$ Trp315-Trp330,  $\gamma$ Trp335-Asn365, and  $\gamma$ Phe295-Thr305. However, using molecular dynamics simulations, it has been shown that during fibrin oligomerization the D:E interface includes binding sites beyond the 'A-a' knob-hole associations, confirming the existence of intermolecular binding sites 'A' and 'a' that are not limited to knobs 'A' and holes 'a' (Zhmurov et al. 2016).

Nevertheless, release of FpA and formation of knobs 'A' are necessary and sufficient to induce fibrin polymerization, resulting in formation of the so-called desA-fibrin. The existence of constitutively open holes 'a' in fibrinogen and fibrin is also essential for fibrin polymerization. If the holes 'a' are obstructed by the GPRP peptide (Everse et al. 1998b) or compromised by a replacement of the most important residue  $\gamma$ Asp364 (Okumura et al. 1997), fibrin polymerization is abrogated. Overall, these and other data suggest that fibrin polymerization and clot formation are driven by the 'A-a' interactions.

### 13.3.4 Fibrin Oligomers and Protofibrils

Fibrin polymerization begins when two fibrin monomer molecules formed after cleavage of FpA interact to form a half-staggered dimer in which knob 'A' binds hole 'a', and there are two 'A-a' knob-hole interactions holding the two monomers together (Erickson and Fowler 1983). A third fibrin molecule added to a half-staggered dimer forms an end-to-end connection where the two adjacent molecules touch each other and the lateral D regions of two molecules form the D:D interface that comprises the junction between monomers in each of two strands in fibrin oligomers (Everse et al. 1998a; Fig. 13.4). The D:D interface comprises residues  $\gamma$ 275–309 (Everse et al. 1998b) and is very weak, because it yields first upon forced stretching of fibrin(ogen) oligomers (Zhmurov et al. 2011). It was found that the D:D interactions involve residues  $\gamma$ 275,  $\gamma$ 308, and  $\gamma$ 309 that are essential for elongation of fibrin strands (Marchi et al. 2006; Bowley et al. 2009). Fibrin monomers can add longitudinally to form longer two-stranded fibrin oligomers of varying length. Lateral interactions between two strands of fibrin oligomers are mediated by the central E region of one fibrin molecule and two lateral D regions of two other molecules (Fig. 13.4). The D-E-D complex is held together mainly by the 'A-a' knob-hole bonds and by additional interactions at the D:E and D:D interfaces (Kononova et al. 2013; Zhmurov et al. in preparation).

Fibrin monomers continue to add longitudinally to the oligomers, which lengthen further to make two-stranded protofibrils (Erickson and Fowler 1983), a critically important intermediate product of fibrin polymerization (Fig. 13.5). Protofibrils are usually about 0.5–0.6  $\mu\text{m}$  in length, which corresponds to  $\sim$ 20–25 monomers, and they are long enough to self-interact and aggregate laterally (Erickson and Fowler 1983; Chernysh et al. 2011). Oligomers and protofibrils have been visualized by transmission electron microscopy (Erickson and Fowler 1983; Medved et al. 1990; Weisel et al. 1993; Chernysh et al. 2011; Huang et al. 2014), light microscopy (Chernysh and Weisel 2008; Chernysh et al. 2011), and atomic force microscopy (Yermolenko et al. 2011; Protopopova et al. 2015). In the presence of the fibrinogen  $\gamma'$ , which is a  $\gamma$  chain splice variant, protofibril formation is partially impaired by likely because of electrostatic repulsion (Cooper et al. 2003; Gersh et al. 2009; Allan et al. 2012).



**Fig. 13.5** Atomic force microscopy images of fibrinogen, fibrin oligomers, and protofibrils and reconstruction of a protofibril model. (a–i). Images by high-resolution atomic force microscopy (Published with permission and thanks to Drs. Anna D. Protopopova, Nikolay Barinov, Dmitry Klinov). All magnification bars = 30 nm. (a–c). Fibrin monomer with visible  $\alpha$ C regions. (d). Fibrin dimer. (e). Fibrin trimer. (f). Fibrin tetramer. (g–h). Fibrin protofibrils. (i). Two protofibrils aggregating laterally. On the left, the two protofibrils are diverging, creating a branch-point. (j). Reconstruction of a twisted fibrin protofibril based on the X-ray crystallographic structure of fibrinogen (PDB Entry: 3GHG). The molecules are shown with addition of missing parts of the crystal structure reconstructed from molecular dynamics simulations, including the full-length  $\alpha$ C regions. ‘A-a’ knob-hole bonds that are the major basis of fibrin polymerization are as in Fig. 13.4 (Published with permission and thanks to Dr. Artem Zhmurov)

### 13.3.5 Lateral Aggregation of Protofibrils

Protofibrils aggregate laterally to form more or less thick fibers, in which the half-staggered molecular packing gives rise to a 22.5-nm periodicity corresponding to half the length of the fibrin molecule (Fig. 13.3). This longitudinally ordered molecular packing of fibrin fibers was visualized by transmission electron microscopy of negatively contrasted specimens and atomic force microscopy as regular cross-striations (Weisel 1986; Yermolenko et al. 2011) (Fig. 13.10b). The mechanisms, structural motifs involved, and driving forces of the protofibrils’ lateral aggregation are mostly still unknown. Importantly, protofibrils self-associate laterally only after they reach a certain threshold length, implying that the bonds mediating the interactions between protofibrils are weak and cooperative along the axis of the protofibril. At present, the structures that have been shown or presumed to participate in inter-protofibril lateral aggregation are the following: knobs ‘B’ and holes ‘b’, the  $\alpha$ C regions, the C-terminal parts of the  $\gamma$  chains, adjacent  $\beta$ -nodules (Yang et al. 2000), the coiled-coils (Okumura et al. 2006), and N-glycans at residues  $\beta$ Asn364 and  $\gamma$ Asn52 (Langer et al. 1988).

Because some other structural proteins that self-assemble have similar intermolecular interactions *in vitro* as *in vivo* (Weisel et al. 1978), useful mechanistic information about lateral aggregation of protofibrils has been gleaned from the interfaces formed between D regions in crystals. Thus, a mechanism for lateral aggregation has been proposed based on crystal packing via direct association of  $\beta$ -nodules of neighboring protofibrils, employing residues  $\beta$ 330–375 (Yang et al. 2000). Some data suggest that lateral aggregation of protofibrils could be mediated by the residues  $\beta$ Ala68 located in the N-terminal portion of the  $\beta$  chain (Mullin et al. 2000) and  $\beta$ Gly15, the N-terminal residue of knob 'B', no matter whether FpB is released or not (Hirota-Kawadobora et al. 2003).

The packing in fibrin generally seems to be paracrystalline (Weisel et al. 1983; Weisel 1986), meaning that fibrin is more regularly organized axially than laterally, but there is also evidence for regularity in lateral packing (Torbet et al. 1981; Caracciolo et al. 2003; Yeromonahos et al. 2010). In addition, it appears that bundling of twisted protofibrils proceeds in such a way that the resulting fibers are also twisted (Medved et al. 1990). To maintain the 22.5-nm repeat, protofibrils that are newly added to the outside of a fiber must be stretched as their path length increases. This may comprise a thermodynamic mechanism to control the diameter of fibers, as the lateral aggregation stops when the protofibril stretching energy surpasses the energy of bonding.

An alternative model of fibrin formation and unusual structure is based on the formation of ultrathin fibrin sheets spanning channels on a plastic substrate, but the physiological relevance of these results is not yet known (O'Brien et al. 2008). Another proposed mechanism of the early stages of fibrin assembly implies that short thin fibrin branches form at an initial phase of polymerization, in which single-bonded "Y-ladder" polymers rapidly elongate before undergoing a delayed transition to the double-stranded fibrils (Rocco et al. 2014).

### 13.3.6 Role of 'B-b' Knob-Hole Interactions

When FpB is cleaved off, the  $\beta$  chain acquires a new N-terminal sequence Gly-His-Arg-Pro (GHRP), comprising knob 'B' capable of binding to hole 'b' located in the  $\beta$ -nodule (Fig. 13.4). The peptide GHRP, which reproduces the structure of knob 'B', has a much lower equilibrium binding affinity for fibrinogen ( $K_d = 140 \mu\text{M}$ ) compared to the peptide GPRP ( $K_d = 25 \mu\text{M}$ ) that mimics the structure of knob 'A' (Everse et al. 1998b). Moreover, a much larger piece of fibrin molecule from the central part (bearing knobs 'A') interacted with fibrinogen (bearing holes 'a') with even higher affinity ( $K_d = 5.8 \pm 1.1 \mu\text{M}$ ; Geer et al. 2007), suggesting that the 'A-a' interactions are not limited to the Gly-Pro-Arg motif. There must be additional interface beyond the 'A-a' bonds that has substantially higher binding strength, which has been confirmed using structural modeling (Kononova et al. 2013; Zhmurov et al. 2016).

Despite direct evidence for the existence of ‘B-b’ interactions (Litvinov et al. 2007a), the role of the ‘B-b’ interactions in fibrin formation is not quite clear because it is supported only indirectly. If fibrin is formed by cleavage of FpA only (while FpB remains uncleaved) the network consists of thinner fibers compared to the fibrin networks formed after cleavage of FpAs and FpBs. These data suggest that in the presence of ‘B-b’ interactions the fibers are thicker due to enhanced lateral aggregation of protofibrils (Blombäck et al. 1978), despite the fact that cleavage of FpB is not necessary for lateral aggregation and fiber formation. There are rare congenital homozygous fibrinogen mutations (fibrinogens Metz and Frankfurt XIII) in which FpB can be cleaved, while FpA cannot, that still form fibrin clots under the action of thrombin at low temperature (Galanakis et al. 1993). Thus, these clots are formed exclusively via ‘B-b’ interactions, indicating that they can actually occur in fibrin. Moreover, recombinant fibrinogen variants without functional holes ‘a’ (due to mutation of the  $\gamma$ Asp364 residue) formed fibrin under the action of thrombin (that cleaved both FpAs and FpBs) but did not form fibrin under the action of reptilase (that cleaved FpAs only; Okumura et al. 2007). On the other hand, fibrinogen without functional hole ‘b’ (due to mutation B $\beta$ Asp432Ala) formed normal fibrin (Bowley et al. 2009). Therefore, ‘B-b’ bonds not only exist physically but they form during fibrin polymerization under some special conditions (e.g., when ‘A-a’ bonds cannot form), but their role in normal fibrin formation remains largely undefined. Using molecular dynamics simulations, structural and thermodynamic characteristics of the ‘B-b’ interactions has been gleaned (Kononova et al. 2013). It has been proposed that ‘B-b’ interactions have effects on the susceptibility of clots to proteolytic digestion (Doolittle and Pandi 2006). Besides the known ‘A-a’ and ‘B-b’ knob-hole interactions, experimental data suggest that the ‘A:b’ interactions are physically possible, while the ‘B:a’ interactions are unlikely to exist (Litvinov et al. 2005, 2007a). Fibrin structure and properties are affected by addition of a knob ‘B’ mimetic combined with polyethyleneglycol, thus indirectly confirming the functional importance of ‘B-b’ knob-hole interactions (Brown et al. 2015).

### ***13.3.7 The Role of the $\alpha$ C Regions in Fibrin Formation***

During fibrin polymerization, the protruding and flexible  $\alpha$ C regions (see Sects. 13.2.4 and 13.2.5) can self-interact both within and between protofibrils; these  $\alpha$ C- $\alpha$ C interactions can lead to formation of  $\alpha$ C polymers that are reinforced with additional crosslinking by Factor XIIIa (Tsurupa et al. 2011; Fig. 13.3). The  $\alpha$ C- $\alpha$ C polymerization involves two mechanisms. One is self-association of the  $\alpha$ C-domains that occurs via their N-terminal subdomains by  $\beta$ -hairpin swapping. The second is the interaction of the C-terminal subdomain with the  $\alpha$ C-connector (Tsurupa et al. 2012). Although they are not necessary, the  $\alpha$ C regions are known to augment lateral aggregation (Weisel and Medved 2001; Litvinov et al. 2007b; Tsurupa et al. 2011). Fibrinogen lacking the  $\alpha$ C regions forms clots with thinner fibers and a higher density of branch points than clots made of full-length fibrinogen (Collet

et al. 2005). Furthermore, a recombinant fibrinogen construct that has the longer human  $\alpha$ C regions replaced with the shorter chicken  $\alpha$ C regions displayed impaired lateral aggregation of protofibrils (Ping et al. 2011).

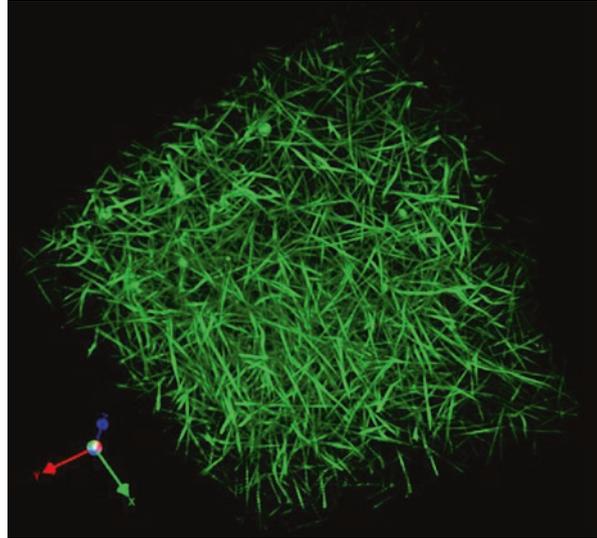
### ***13.3.8 Fibrin Branching and Network Architecture***

As fibrin fibers thicken by lateral aggregation and grow in length, they also branch, yielding a space-filling 3D network. Electron microscopy revealed at least two different molecular mechanisms by which branch points may form (Figs. 13.3 and 13.5i). One of them known as a “bilateral junction”, originates from two protofibrils that undergo incomplete lateral aggregation but diverge into two separate protofibrils, each of them giving rise to a new fiber (Mosesson et al. 1993). The second type of branchpoint, called a “trimolecular junction” or “equilateral junction”, forms when a fibrin monomer attached to the end of a protofibril via only one ‘A-a’ bond (or one  $\gamma$ -nodule), such that both the monomeric molecule and the protofibril to which it is bound can grow independently, forming two strands each (Fogelson and Keener 2010). In either case, most of branch points in clots consist of three fibers of about the same diameters joined together (Ryan et al. 1999), suggesting that the type of initial branchpoint does not affect much the final network structure. Branching has been observed directly in an imaging study of the early stages of fibrin polymerization (Chernysh et al. 2011). Finally, an increase in the number of branch points in a clot normally correlated with a decrease of the fiber diameters (Ryan et al. 1999). The aggregate of data support a notion that that branching (as a part of elongation process) and lateral aggregation compete. In other words, the prevalence of lateral aggregation leads to fibrin with thicker fibers and fewer branch points, while reduced lateral aggregation will result in formation of fibrin with thinner fibers and more branch points.

### ***13.3.9 Fibrin Structure and the Gelation Point***

Formation of a fibrin clot is a transition from sol to gel upon formation of a three-dimensional filamentous network (Fig. 13.6). The gelation point determined as blood/plasma clotting time is commonly used in clinical assays as a test to reveal coagulation disorders. The gelation point occurs when only about 15–20 % of the fibrinogen is converted to fibrin, which is enough to form the gel (Chernysh and Weisel 2008). Some correlations between gelation time and final clot structure have been established (Blombäck and Okada 1982), but the formation of a stable fully branched network is not yet completed at the gelation point, so that new fibers and branch points continue to form in the gel (Chernysh and Weisel 2008). The structure of fibrin networks can be determined and quantified using scanning electron microscopy by a number of parameters, such as the fiber diameter, fiber density, number of

**Fig. 13.6** Fibrin clot network. A 3-dimensional reconstruction of a hydrated fibrin gel obtained using fluorescent confocal microscopy. Fibers are very straight under tension and branch to form a network. Fibrin(ogen) was fluorescently labeled with Alexa 488 (Brown et al. 2009)



branch points, fiber length, and the size of the pores (Fig. 13.10a). All of these parameters are variable and depend on the kinetics of fibrin polymerization. Various biophysical techniques have been applied to study the fine structure of fibrin clots that revealed the complex structural hierarchy at different spatial scales (Ryan et al. 1999; Ferri et al. 2002; Guthold et al. 2004; Evans et al. 2010; Yeromonahos et al. 2010; Magatti et al. 2013). Remarkably, the diffusion rate of proteins in fibrin clots does not depend much on the structure of the network because there are large pores. However, permeability of fibrin clots for fluid (Okada and Blombäck 1983) or nanoparticles (Spero et al. 2011) perfused through the clot depends strongly on the pore size and hence on overall clot structure. The diffusivity of the fibrin network is reduced dramatically when it is embedded with blood cells and compressed, as happens in a contracted whole blood clot (Cines et al. 2014).

### 13.3.10 *Factor XIIIa-Catalyzed Covalent Crosslinking of Fibrin*

To stabilize the clot against proteolytic and mechanical insults, fibrin is covalently crosslinked by the plasma transglutaminase, Factor XIIIa, an active form of Factor XIII zymogen activated by thrombin in the presence of  $\text{Ca}^{2+}$ . The C-terminal ends of the  $\gamma$  chains of fibrin(ogen) have amino acid residues comprising a crosslinking site for two end-to-end interacting molecules that form covalent isopeptide  $\epsilon$ -( $\gamma$ -glutamyl)-lysyl bonds between the  $\gamma\text{Lys}406$  of one molecule and  $\gamma\text{Gln}398/399$  of another molecule (Fig. 13.7). There has been a disagreement about longitudinal  $\gamma$ - $\gamma$ -crosslinking within a strand of a protofibril versus transverse  $\gamma$ - $\gamma$ -crosslinking



Domingues et al. 2015; Muthard et al. 2015). An increase in plasma fibrinogen  $\gamma'$  concentration is associated with the risk of myocardial infarction and other thrombotic states (Mannila et al. 2007; Lovely et al. 2002; Uitte de Willige et al. 2005, 2009). Fibrinogen polymorphisms with functional consequences can occur in the polypeptide chains other than the  $\gamma$  chain.  $\alpha E$  fibrinogen (see Sect. 13.2.1) has a reduced rate of fibrin polymerization and forms thinner and more branched fibers than fibrin networks containing the  $\alpha$  chains (Mosesson et al. 2004). The fibrinogen  $A\alpha$  chain gene *FGA* polymorphism 2224G/A has been associated with reduced clot permeability (Mannila et al. 2006), while the Ala312 allele of *FGA* 6534/Thr312Ala was associated with increased clot stiffness (Standeven et al. 2003), and the Lys448 allele of the fibrinogen  $B\beta$  chain gene (*FGB*) polymorphism  $B\beta$ Arg448Lys resulted in a compact fibrin network structure resistant to lysis (Ajjan et al. 2008). The other most widespread polymorphisms in the fibrinogen genes occur in the noncoding regions and can result in changes in plasma fibrinogen levels. There are many more examples of strong associations between fibrinogen polymorphisms, clot structure and properties, and disease (Ariëns et al. 2002; Scott et al. 2004).

### 13.4.2 *Post-translational Modifications and Heterogeneity of Fibrinogen*

There are many molecular forms of fibrinogen present in blood, as originally detected from variations in biochemical properties and gel electrophoretic behavior. It has been estimated that fibrinogen may occur in more than a million non-identical forms in a healthy individual as a result of the many combinations of modified or inherently polymorphic sites (Henschen-Edman 2001). Several of these genetically determined variations have already been mentioned, but there are also post-translational heterogeneities originating from multiple biochemical reactions that accompany various physiological and especially pathological conditions, such as inflammation or ischemia. These reactions can modify the fibrinogen molecule in many ways, such as phosphorylation at specific seryl and threonyl sites, prolyl hydroxylation, tyrosyl sulfation, asparaginyl or glutaminyl deamidation, N-terminal pyroglutamate formation from glutaminyl precursors, oxidation of methionine, histidine and tryptophan residues, tyrosine nitration, modifications of cysteine residues, formation of dityrosine and carbonyl groups, etc. The C-terminal portion of the  $A\alpha$  chain is especially susceptible to limited cleavage by intracellular and extracellular proteolytic enzymes, but some digestion also occurs at specific sites in the  $B\beta$  and  $\gamma$  chains, such that lower molecular weight forms are commonly present in plasma fibrinogen. Alternative N-glycosylation can be another source of fibrinogen heterogeneity because it may result in formation of oligosaccharides with a variable structure (Brennan 2015). Acquired abnormal fibrinogen variants occur in patients with several conditions via non-enzymatic reactions, including glycation of lysine residues in uncompensated diabetes mellitus (Dunn and Ariens 2004) or

homocysteinylation in hyperhomocysteinemia (Sauls et al. 2003). Fibrinogen derivatives can form in pathological conditions associated with thrombin generation, activation of fibrinolytic enzymes, or immune reactions, for example crosslinked fibrin(ogen) degradation products form when fibrinolytic activity in the blood is excessive, and antibody-fibrinogen complexes are formed in some autoimmune diseases. Individuals treated with acetylsalicylic acid (aspirin) have acetylated lysine residues (Ajjan et al. 2009). Oxidative stress has been widely implicated in physiological processes such as aging, in various disease pathogenesis, including arterial and venous thrombosis. Proteins are major targets for oxidants, and fibrinogen is a common target for oxidative post-translational modifications (Martinez et al. 2013; Rosenfeld et al. 2014). Neutrophils and monocytes, the most prevalent leukocytes in the sites of inflammation and venous thrombi, generate nitrating metabolic intermediates capable of nitration of fibrinogen (Heffron et al. 2009; Martinez et al. 2012). Tobacco smokers tend to have nitration of tyrosine and oxidation of methionine, histidine or tryptophan residues (Parastatidis et al. 2008). Many of these chemically modified forms are associated with differences in functional and structural properties of fibrinogen and fibrin, including a thrombogenic phenotype associated with increased risk of arterial and venous thrombosis (Nowak et al. 2007; Parastatidis et al. 2008; Paton et al. 2010; Sauls et al. 2006; Undas et al. 2006; Weigandt et al. 2012).

### ***13.4.3 Hereditary Fibrinogen Defects (Dysfibrinogenemias, Afibrinogenemia, and Hypofibrinogenemia)***

Dysfibrinogenemias are characterized by inherent structural changes in the fibrinogen molecule that commonly result in alterations in clotting or other functional aspects of the protein (Casini et al. 2016). Traditionally, particular defective fibrinogen variants are named after the city of their discovery or where the patient lived. Most congenital defects are rare but have led to important insights into fibrin(ogen) structure-function relationships. Many of these mutations have been described in reviews (Galanakis 1993; Roberts et al. 2001; Matsuda and Sugo 2001; de Moerloose and Neerman-Arbez 2009; Asselta et al. 2006; de Moerloose et al. 2013; Asselta et al. 2015; Casini et al. 2015). In addition, the website, <http://www.geht.org/databaseang/fibrinogen>, maintains a database of fibrinogen mutations and their functional consequences.

Mutations that give rise to dysfibrinogenemias are commonly caused by single base mutations that lead to the substitution of a single amino acid residue, but other mutations can give rise to a stop codon, resulting in a truncation of one of the chains. In addition, base additions or deletions may occur, with consequences for fibrinogen structure and function. Such mutations can cause a predisposition to thrombosis, or bleeding, or be asymptomatic, depending on their functional effects. They can affect fibrinopeptide cleavage, fibrin polymerization, Factor XIIIa-catalyzed crosslinking,

susceptibility to fibrinolysis or integrin  $\alpha$ IIb $\beta$ 3-mediated platelet aggregation. In homozygous forms of the dysfibrinogenemias, the mutations occur in all fibrinogen molecules so only abnormal homodimers are present in the blood, but these mutations are rare, so most dysfibrinogenemias are heterozygous. As a result, the pool of fibrinogen molecules in most subjects with a dysfibrinogenemia consists of various proportions of normal homodimers, mutant homodimers and heterodimers.

Most dysfibrinogenemias are likely to be undetected by clotting assays, but some of the most commonly found alterations are in the N-terminal part of the A $\alpha$  chain, in the regions responsible for thrombin binding or cleavage or in or near knob 'A', i.e. the A $\alpha$ Gly17-Pro18-Arg19 motif. One such mutation is a substitution of A $\alpha$ Arg16 (the last residue of FpA) by His or Cys, the former causing delayed release of FpA, with the latter completely abrogating its release. With no FpA cleavage, FpB is released slowly and clots made of desB-fibrin form, but only at lower temperatures (Shainoff and Dardik 1979). Mutations of A $\alpha$ Gly17, A $\alpha$ Pro18, A $\alpha$ Arg19 or A $\alpha$ Val20 result in defective polymerization because of alterations of knobs 'A.'

Mutations of the B $\beta$  chain are less common. Substitution of Cys for B $\beta$ Gly at position 15 results in slow polymerization from delayed release of FpB (Sugo et al. 2000). Mutation of B $\beta$ Ala68 to Thr results in defective binding of thrombin to fibrin and consequent thrombosis (Koopman et al. 1992), since fibrin is a physiological absorbent of thrombin and was initially called antithrombin I (Mosesson 2007).

In the  $\gamma$  chain, many mutations in the C-terminal portion have been identified, particularly at position  $\gamma$ Arg275 (Cote et al. 1998). This residue makes contacts at the D:D interface, so that mutations affect fibrin polymerization. Mutations in or near the hole 'a' are common but patients with most of these substitutions are asymptomatic, presumably because they are heterozygous.

Some point mutations are responsible for congenital hypo- and afibrinogenemia, as a result of defects in molecular processing, assembly, secretion, and domain stability of fibrinogen. Afibrinogenemia is an autosomal recessive disorder characterized by the complete absence of detectable fibrinogen in the blood and hypofibrinogenemia is characterized by a reduced level of fibrinogen. Analysis of the three fibrinogen genes in affected individuals has led to the identification of several causative mutations (Brennan et al. 2001; Neerman-Arbez 2001), mostly as a result of mutations in the *FBA* gene. The analysis of the degree of severity of the fibrinogen disorders associated with truncation of the A $\alpha$  chain suggest that formation of the distal disulfide ring of the coiled-coil is essential for assembly and secretion of fibrinogen molecules.

### 13.4.4 Environmental Conditions of Fibrin Formation

Fibrin formation, structure, and properties both *in vitro* and *in vivo* are strongly affected by external factors, such as ionic strength, composition, pH and various endogenous and exogenous substances, e.g., polyphosphate, mono-, oligo- and polysaccharides, peptides, lipids, proteins, nucleic acids, medications, as well as many other normal and pathological, natural and artificial compounds present in

blood and injured tissues. Thrombin activity in blood has a profound effect on fibrin, with a high thrombin activity resulting in clots with thinner fibers, a higher density of branch points, and smaller pores, while a low thrombin activity results in thicker fibers with fewer branch points and larger pores. Most of these structural variations are based on the kinetics of individual steps of fibrin polymerization (Weisel and Nagaswami 1992). The structure of fibrin networks is often affected by physical factors, such as hydrodynamic flow or a strong magnetic field that result in formation of oriented anisotropic fibrin fibers (Gersh et al. 2010; Campbell et al. 2010). Fibrin structure and properties are greatly influenced by the presence of blood cells, namely activated platelets and erythrocytes (Aleman et al. 2014; Malecki et al. 2015) that form a natural and very active environment for clot formation. In addition to whole cells, the effects of circulating cell-derived microparticles on the fibrin clot structure and properties have been recently demonstrated (Zubairova et al. 2015). Fibrin can interact with other components of the extracellular matrix, both filamentous and non-filamentous, that not only affects fibrin structure but imparts additional mechanical and chemical stability (Maquart and Monboisse 2014). The structure of fibrin clots can be directly related to clinical conditions associated with thrombosis (Collet et al. 2006; Zalewski et al. 2015).

Here is a list of plasma proteins that bind specifically to fibrinogen or fibrin or both, with some indications of the biological significance:

<i>Actin</i>	modulation of clot structure and properties (Talens et al. 2012)
<i>Albumin</i>	modulation of lateral aggregation and clot structure (Galanakis et al. 1987; Talens et al. 2012)
<i><math>\alpha</math>1-Antitrypsin</i>	local regulation of proteases involved in coagulation or fibrinolysis (Talens et al. 2012)
<i><math>\alpha</math>2-Antiplasmin</i>	local regulation of fibrinolysis (Tsurupa et al. 2010)
<i>Apolipoproteins A-IV, A-I, J, E</i>	associated with high-density lipoproteins; the physiologic role of binding to fibrin(ogen) is not clear (Talens et al. 2012)
<i>Carboxypeptidase N CD44</i>	a local fibrinolysis inhibitor (Talens et al. 2012) mediates tumor metastasis at the sites of fibrin deposition (Alves et al. 2009)
<i>Coagulation Factor Xa</i>	a negative feedback for thrombin generation (Iino et al. 1995)
<i>Coagulation Factor VIII</i>	platelet-attached soluble fibrin mediates binding of Factor VIII (Gilbert et al. 2015)
<i>Coagulation Factor XIII</i>	crosslinking of fibrin and multiple plasma proteins (Weisel and Dempfle 2013)
<i>Complement C3</i>	delayed fibrinolysis (Howes et al. 2012)

<i>Factor H-related proteins-associated lipoprotein particles (FALP)</i>	the physiologic role of fibrinogen on FALP is not clear (Park and Wright 2000)
<i>Ferritin</i>	some fibrinogens circulate in the form of a complex with ferritin (Takahashi et al. 2013)
<i>Fibroblast Growth Factor-2</i>	augmented angiogenesis and cell proliferation at the sites of fibrin deposition (Sahni et al. 1999)
<i>Fibulin</i>	interferes with the fibrin assembly (Tran et al. 1995)
<i>Haptoglobin</i>	associated with high-density lipoproteins; the physiologic role of binding to fibrin(ogen) is not clear (Talens et al. 2012)
<i>Hepatocyte-derived fibrinogen related protein-1</i>	liver cell growth regulation (Talens et al. 2012)
<i>Immunoglobulins</i>	modulate fibrin structure, localize immune and inflammatory reactions (Talens et al. 2012)
<i>Interleukin-1<math>\beta</math></i>	modulation of inflammation (Sahni et al. 2004)
<i>Lipoprotein(a)</i>	modulation of fibrinolysis (Weisel and Litvinov 2014)
<i><math>\alpha</math>2-Macroglobulin</i>	local regulation of proteases involved in coagulation or fibrinolysis (Talens et al. 2012)
<i>Myosin</i>	modulation of fibrinolysis (Kolev et al. 2003)
<i>Plasma fibronectin</i>	platelet adhesion and thrombus formation (Gailit and Ruoslahti 1988; Talens et al. 2012)
<i>Plasminogen</i>	promotion of fibrinolysis (Weisel and Litvinov 2014)
<i>Plasminogen activator inhibitor-1</i>	modulation of fibrinolysis (Smolarczyk et al. 2005)
<i>Serum amyloid P</i>	associated with high-density lipoproteins; the physiologic role of binding to fibrin(ogen) is not clear (Talens et al. 2012)
<i>Thrombin</i>	elimination of active thrombin from the circulation (Weisel and Dempfle 2013)
<i>Thrombin-activatable fibrinolysis inhibitor (TAFI)</i>	modulation of fibrinolysis (Valnickova and Enghild 1998)
<i>Thrombospondin</i>	modulation of fibrinolysis (Bacon-Baguley et al. 1990)
<i>Tissue-type plasminogen activator (t-PA)</i>	promotion of fibrinolysis (Medved et al. 2001)
<i>Vascular Endothelial Growth Factor (VEGF)</i>	promotion of angiogenesis at the sites of fibrin deposition (Sahni and Francis 2000)
<i>VE-cadherin</i>	suppression of inflammation by inhibiting leukocyte transmigration at the sites of fibrin deposition (Yakovlev et al. 2011)

<i>Very low density lipoprotein receptor (VLDLR)</i>	promotion of inflammation and transendothelial migration of leukocytes at the sites of fibrin deposition (Yakovlev and Medved 2015)
<i>Vitronectin</i>	platelet adhesion and thrombus formation (Schvartz et al. 2002; Podor et al. 2002)
<i>von Willebrand Factor</i>	platelet adhesion and thrombus formation (Miszta et al. 2014)

### 13.4.5 Fibrin Formation Under Hydrodynamic Flow

Blood flow is one of the most important physical factors that affects profoundly formation of the fibrin network, its structure and properties *in vivo* (Swieringa et al. 2016). Clot formation in static conditions stops when all soluble fibrinogen is converted to insoluble fibrin. Therefore, in normal human plasma with a fibrinogen concentration of about 3 g/L, a fibrin clot will consist of only 0.3 % protein and 99.7 % liquid by mass. Alternatively, under flow conditions more fibrinogen is added to the forming clot, so the clot will contain more protein and have a different structure with much denser, thicker and bundled fibers (Silvain et al. 2011; Neeves et al. 2010). Fibrin formed under flow conditions also has some fibrin fibers orientated along the direction of flow (Gersh et al. 2010; Campbell et al. 2010; Whittaker and Przyklenk 2009), which changes clot mechanical properties and affects its susceptibility to enzymatic lysis (Campbell et al. 2010; Varju et al. 2011). Furthermore, it has been proposed from microfluidics experiments that the shear forces of blood flow determine the likelihood of embolization, namely the rupture of a piece of a clot that is carried by the blood stream to block another vessel (Colace et al. 2012; Brass and Diamond 2016).

## 13.5 Fibrin Mechanical Properties and Their Structural Origins

### 13.5.1 General Remarks

Fibrin mechanics is an important and rapidly developing field because fibrin networks form at sites of vascular injuries and perform a mechanical task of stemming blood flow by forming a gel under hydrodynamic blood shear (Campbell et al. 2010; Flamm and Diamond 2012), contraction of platelets (Lam et al. 2011), contraction of adjacent muscles, and pulsation of a vessel wall (Gasser et al. 2008; Ashton et al. 2009). Therefore, the outcomes of many bleeding and thrombotic disorders, including thromboembolism, are largely determined by the mechanical behavior of fibrin networks (Tran et al. 2013). In addition, fibrin has been used as a biomaterial for

numerous purposes of surgical repairs and tissue engineering to stop or control bleeding or to form a provisional fibrin matrix for growing blood vessels and tissue regeneration, all of which strongly depend on the mechanical properties of fibrin gels.

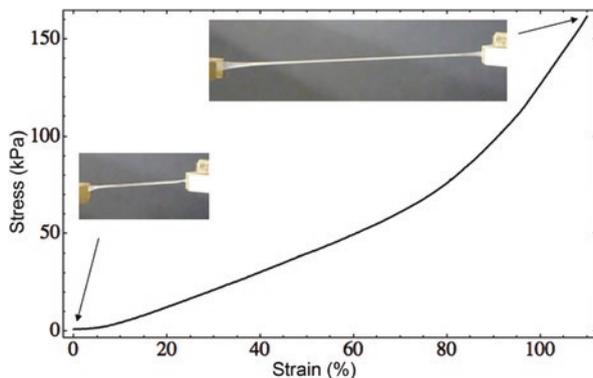
### ***13.5.2 Viscoelastic Properties of Fibrin***

Fibrin is a viscoelastic polymer, which means that it has both elastic and viscous properties. The elasticity (or stiffness) is characterized by reversible mechanical deformation, while viscosity (or plasticity) is characterized by a slow irreversible deformation (creep) induced by force. Viscoelastic biomaterials differ in the relative degrees of both elastic and viscous properties. The elastic response of the fibrin clot is characterized by the shear storage modulus,  $G'$ , corresponding to the part of shear stress that is in phase with strain. The viscous response of the clot to applied shear is measured by the shear loss modulus,  $G''$ , where strain lags stress. The storage and loss moduli determine how the clot responds to the forces to which it is subjected. The ratio  $G''/G'$  is often used to characterize relative viscosity and stiffness of a fibrin clot.

Clots derived from the blood of subjects with pulmonary embolism showed accelerated establishment of viscoelastic properties compared to healthy donors (Martinez et al. 2014). In addition, the stiffness of clots formed from the blood of patients who have had heart attacks at an early age is 50 % greater than that of controls (Collet et al. 2006). The clot fractal dimension, based on viscoelastic properties of incipient blood clots, has been used as a biomarker of prothrombotic clot microstructure (Lawrence et al. 2015; Davies et al. 2015). Platelets sense the stiffness of the underlying fibrin/fibrinogen substrate so that higher substrate stiffness leads to increased platelet activation, adhesion and spreading (Qiu et al. 2014; Wufsus et al. 2015). Lastly, the stiffness of the fibrin scaffold of occlusive thrombi is a major determinant for effectiveness of their mechanical damage and removal to restore the impaired blood flow (Weiss et al. 2013).

### ***13.5.3 Non-linear Elasticity and High Extensibility of Fibrin***

The elasticity of fibrin clots is generally characterized by a stress-strain curve, in which an applied stress (force/area) is plotted against the degree of induced deformation (strain) (Fig. 13.8). At low strains or deformations of fibrin, stress is directly proportional to strain and the slope of the curve (the elastic modulus) is constant. At larger strains, the linearity is broken and the slope of the curve increases dramatically, so that the elastic modulus or stiffness of the clot increases up to one order of magnitude or more (Janmey et al. 1983). This non-linearity is called strain hardening or strain stiffening and is a fundamental property of biological gel-like structures (Storm et al. 2005).



**Fig. 13.8** Stress-strain curve of a fibrin clot. Representative stress-strain curve of a cylindrical fibrin clot reaching greater than a two-fold longitudinal stretch. As the strain increases, the stress on the clot increases linearly until a strain of ~80 % is reached, at which point the sample hardens and enters a new regime with a steeper slope, corresponding to increased stiffness or strain hardening or stiffening. *Insets show photographs of the initial clot and stretched clot*

Fibrin is a highly extensible polymer, which means that under stress blood clots will tend to stretch rather than break. Plasma clots stabilized with Factor XIIIa could be stretched to over three times their relaxed length before breaking (Brown et al. 2009). The extraordinary extensibility, unusual viscoelasticity, including strain stiffening, has been demonstrated and quantified at the level of individual fibers. This can be observed when a fibrin fiber is laterally stretched with a tip of an atomic force microscope, so that crosslinked and uncrosslinked fibrin fibers are stretched to about 2.5 and 3.3 times their original length before rupturing (Liu et al. 2006, 2010; Guthold et al. 2007). The propagation of strain-stiffening throughout the entire fibrin gel is largely determined by the fine structure and non-linear elastic properties of individual fibrin fibers (Hudson et al. 2015; Piechocka et al. 2010). As fibers are stretched, they become stiffer than any surrounding fibers at lower strains, which allow the more strained, stiffer fibers to distribute the strain load to the less strained fibers and reduce strain concentrations (Hudson et al. 2010). In addition to shear and tension, the non-linear elasticity of fibrin has been observed also in response to compressive deformations (Kim et al. 2014).

There are a number of environmental factors that modulate fibrin stiffness both *in vitro* and *in vivo*. The main physiological modulator of fibrin mechanics is Factor XIIIa, which catalyzes fibrin crosslinking (Fig. 13.7) and increases the elastic modulus of fibrin several-fold, apparently by fiber compaction (Kurniawan et al. 2014). Based on the *in vitro* effects of zinc that reduces fibrin clot stiffness, it was suggested that zinc released from activated platelets may modulate clot strength and stability in cooperation with Factor XIIIa. Fibrin elasticity can be modulated by other physical influences (Kotlarchyk et al. 2011; Munster et al. 2013) or biochemical modifications as well as by blood components and cells incorporated into the

fibrin network (Rojas et al. 2009; Weigandt et al. 2012; Lauricella et al. 2013; Jansen et al. 2013; Henderson et al. 2015).

The tunable non-linear elasticity of fibrin may be important biologically because it allows fibrin clots to be compliant at smaller strains and then become stiffer at larger deformations that could otherwise threaten clot integrity and make them prone to embolization. In addition, the complex mechanical behavior of fibrin is important for the interaction between cells and extracellular matrix (Wen and Janmey 2013). Since mechanical stress makes fibrin more resistant to fibrinolysis (Varju et al. 2011; Bucay et al. 2015), fibrin elasticity may be a significant determinant of susceptibility of clots and thrombi to enzymatic lysis (Rottenberger et al. 2013; Longstaff et al. 2013).

### ***13.5.4 Multiscale Structural Mechanics of Fibrin Clots***

It has been shown that fibrin mechanics are governed by a structural hierarchy, implying that fibrin deformation is accompanied by multiple structural rearrangements at different scales, namely the molecular level, individual fibers, fiber network, and the whole clot (Brown et al. 2009; Purohit et al. 2011; Piechocka et al. 2010; Yeromonahos et al. 2010).

At the macroscopic scale ( $10^{-2}$  m), in addition to their large extensibility, fibrin clots also display a dramatic decrease in volume when they are stretched (Brown et al. 2009). The shrinkage of the stretched clot is due to water expulsion and network densification, as confirmed by an approximately tenfold increase in the protein content in clots stretched threefold. This observation might be related to the phenomenon of negative normal stress observed for networks of semiflexible polymers (Guo et al. 2009). An alternative explanation is that the volume change is associated with a molecular structural transition that occurs in stretched fibrin fibers (Brown et al. 2009; Purohit et al. 2011).

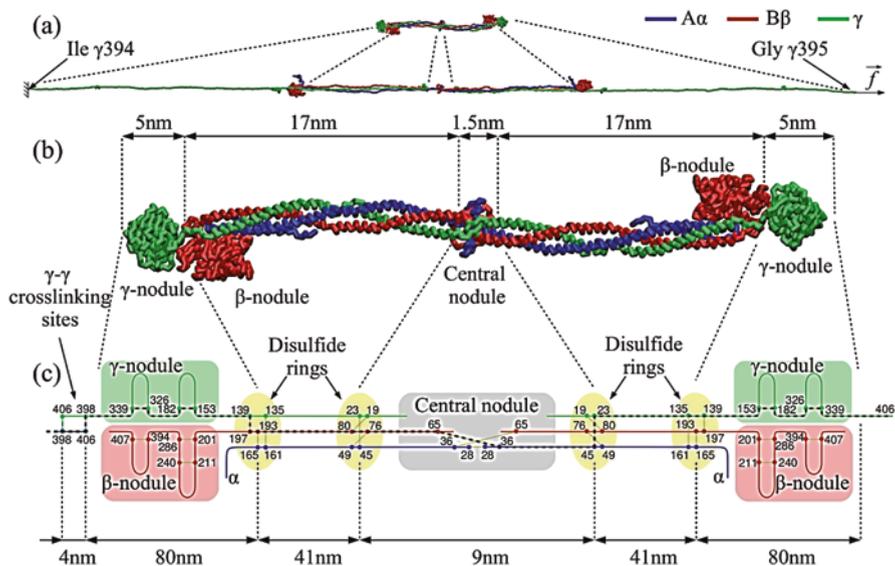
At the network scale ( $10^{-5}$  m), when strain is applied, the fibers begin to align along the direction of strain (Brown et al. 2009; Litvinov et al. 2012; Purohit et al. 2011), the fibers become thinner, closer together, and bundle (Müller et al. 1984; Brown et al. 2009). A dramatic rearrangement of the fibrin network was also observed in response to compressive deformation (Kim et al. 2014): the fibrin network density increased, fibers reoriented in the compression plane and shorter fiber segments were formed as a result of fiber crisscrossing. These transformations of the network structure are accompanied by a dramatic increase of fibrin elasticity.

At the fiber scale ( $10^{-6}$  m), in response to compression or shear, individual fibrin fibers in the network begin to buckle and bend in the direction of deformation (Lindstrom et al. 2013; Kim et al. 2014). Because buckling and subsequent bending make fibers more compliant, the stiffness of the network in the transverse shear direction gradually decreases with compression.

### 13.5.5 Molecular Structural Origins of Fibrin Mechanical Properties

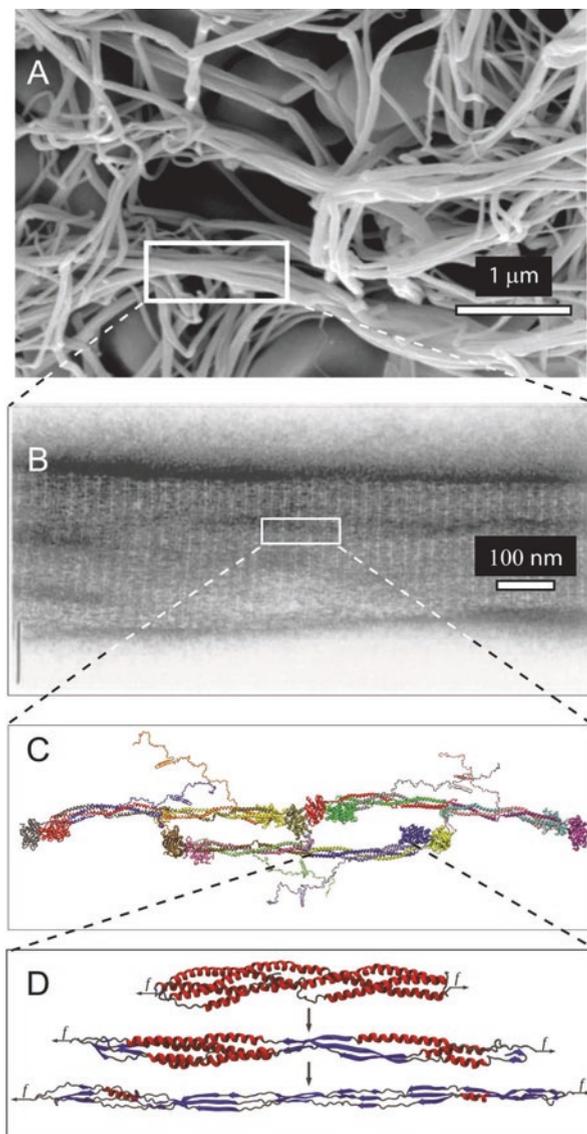
At the molecular level, fibrin has the potential to elongate more than 5-fold, from 45 nm to about 250 nm (Fig. 13.9). The most important structural changes during fibrin deformations include unfolding of coiled-coils (Brown et al. 2007; Lim et al. 2008; Litvinov et al. 2012), the  $\gamma$ -nodules (Averett et al. 2008, 2009), and extension of the  $\alpha$ C regions. In addition, other studies point to a role of network branch points (Carlisle et al. 2010) and the  $\gamma$ A/ $\gamma'$  splice variants (Gersh et al. 2009; Allan et al. 2012). Based on the known crystal structure of a folded fibrin(ogen) molecule (Fig. 13.9), it could be predicted that full hypothetical unfolding of compact structures would result in a  $\sim$ 4.7-fold elongation (Zhmurov et al. 2011; Hudson et al. 2013), which characterizes a great potential contribution of molecular unfolding in large deformations of fibrin.

The first direct experimental observation for an  $\alpha$ -helix to  $\beta$ -strand conversion of the coiled-coils accompanying extension of fibrin was wide-angle X-ray scattering of squeezed fibrin films (Bailey et al. 1943). Unlike the extensively studied  $\alpha$ -helix to  $\beta$ -strand transition in keratin, this preliminary result was not pursued until



**Fig. 13.9** Unfolding of fibrin(ogen). A schematic representation of the fibrin(ogen) molecule in the naturally folded (b) and fully unfolded states ((a) and (c)). The molecule is constrained at the C-terminal part of one  $\gamma$  chain, and mechanical force is applied to the C-terminal part of the other  $\gamma$  chain. (a) Shows full unfolding without much detail, while the structural details are given in (c), showing schematically the lengths of the central nodule,  $\gamma$ -nodules,  $\beta$ -nodules, coiled-coils, taking into account the disulfide bonds. Dimensions are shown in the compact crystal structure (b), and the contour lengths of various structural elements are shown in the fully unfolded state (c), assuming a contour length per residue of 0.38 nm (Zhmurov et al. 2011, with permission of Elsevier Ltd.)

**Fig. 13.10** Unfolding of the coiled-coils of fibrin. (a). scanning electron micrograph of a fibrin clot, with a box enclosing part of a fiber. (b). A transmission electron micrograph of a negatively contrasted fibrin fiber showing the ultrastructure, with the 22.5 nm repeat arising from the half-staggering of 45-nm molecules. (c). A fibrin trimer from X-ray crystallographic data and molecular dynamics simulations of regions not present in the crystal structure. (d). Fibrin  $\alpha$ -helical coiled coils undergoing a forced transition from  $\alpha$ -helix to  $\beta$ -sheet. The mechanical transition from  $\alpha$ -helical coiled coils to  $\beta$ -sheets in the fibrin(ogen) molecule was characterized using molecular dynamics simulations of their forced elongation and theoretical modeling (Adapted with permission from Zhmurov et al. 2012. Copyright 2012 American Chemical Society)



recently. The secondary structure changes during deformations of fibrin polymers studied using Fourier Transform infrared spectroscopy showed that both extension and compression of a hydrated fibrin clot are accompanied by the transition of a triple  $\alpha$ -helix to  $\beta$ -sheet (Litvinov et al. 2012). This structural transition has been confirmed and analyzed in detail using full-atom molecular dynamics simulations of extensional mechanical unfolding of a fibrin(ogen) molecule (Zhmurov et al. 2012; Fig. 13.10).

Based on the paracrystalline molecular packing within a fibrin fiber that results in a 22.5-nm periodicity, it has been hypothesized that forced elongation and unfolding of fibrin molecules during stretching must result in an increase of this repeat. However, small angle X-ray scattering revealed that the position of the peak corresponding to the 22.5-nm spacing does not change significantly as the clot is stretched. Instead, there was an increase in sample disorder, consistent with an increasing number of molecules unfolding non-uniformly in response to the large strain, as expected for a two-state system in which some molecules extend completely while others remain folded (Brown et al. 2009).

Single-stranded fibrinogen oligomers have been prepared and the unfolding of fibrinogen domains has been measured by single-molecule atomic force microscopy (Brown et al. 2007; Zhmurov et al. 2011). In addition, computer-based modeling of the experimental data enabled the visualization of the various structural transitions and identification of relationships to the forces observed in the experimental or simulated force-extension curves. Molecular elongation of fibrin(ogen) is largely determined by the combined sequential unfolding transitions in the C-terminal  $\gamma$  chain nodules and limited reversible extension-contraction of the  $\alpha$ -helical coiled-coil connectors. The coiled-coils act as molecular springs to take up the slack as other domains unfold.

The role of the  $\alpha$ C polymers in fibrin mechanics, underestimated in the past, has been studied intensively more recently (Houser et al. 2010; Falvo et al. 2010; Ping et al. 2011; Helms et al. 2012; Averett et al. 2012). It has been shown that the transglutaminase-catalyzed covalent crosslinking of the  $\alpha$  chains contributes substantially to the fibrin clot stiffness and elasticity (Collet et al. 1999; Piechocka et al. 2010; Helms et al. 2012; Duval et al. 2014; Kurniawan et al. 2014), including the fast elastic recoil of stretched fibrin fibers (Hudson et al. 2013). Based on the exceptional role of the  $\alpha$ C regions in fibrin mechanics, a structural model of fibrinogen has been proposed that captures the stress-strain behavior of individual fibrin fibers (Averett et al. 2012).

### ***13.5.6 Fibrin as a Biomaterial***

A new impetus to study fibrin mechanics came from its biomedical applications as a hydrogel (Janmey et al. 2009). Fibrin has been extensively used as a biopolymer scaffold in tissue engineering, taking advantage of its unique biological and physical characteristics, including its porosity, deformability, elasticity and biodegradability (Ahmed et al. 2008). Fibrin alone, or in combination with other materials, has been used as a biological scaffold for stem or primary cells to regenerate adipose tissue, bone, cardiac tissue, cartilage, liver, nervous tissue, ocular tissue, skin, tendons, and ligaments. Although fibrin can be used as the only biomaterial, it has been shown that the addition of collagen to fibrin can provide extra stiffness and durability of the forming biomaterial (Lai et al. 2012). Gels based on fibrin, alone or in combination with gelatin, collagen, or elastin, have been used to optimize cellular

activities, including differentiation, proliferation, and changes in morphology (Shevchenko et al. 2010; Chaterji et al. 2007; Dikovsky et al. 2006; Natesan et al. 2011; Kuehn et al. 2014; Brougham et al. 2015). The ability of cells to modify their phenotype and behavior in response to variations in the stiffness of a fibrin-containing substrate (called mechanosensing) has been used, e.g., in regulation of neurite outgrowth in fibrin gels (Man et al. 2011) and modulation of the secretory activity of endothelial cells and mesenchymal stem cells (Rao et al. 2012). Fibrin clots with longitudinally aligned fibrin fibers could serve as a scaffold for longitudinal axonal regrowth in cases of traumatic peripheral nerve injuries (Gessmann et al. 2016).

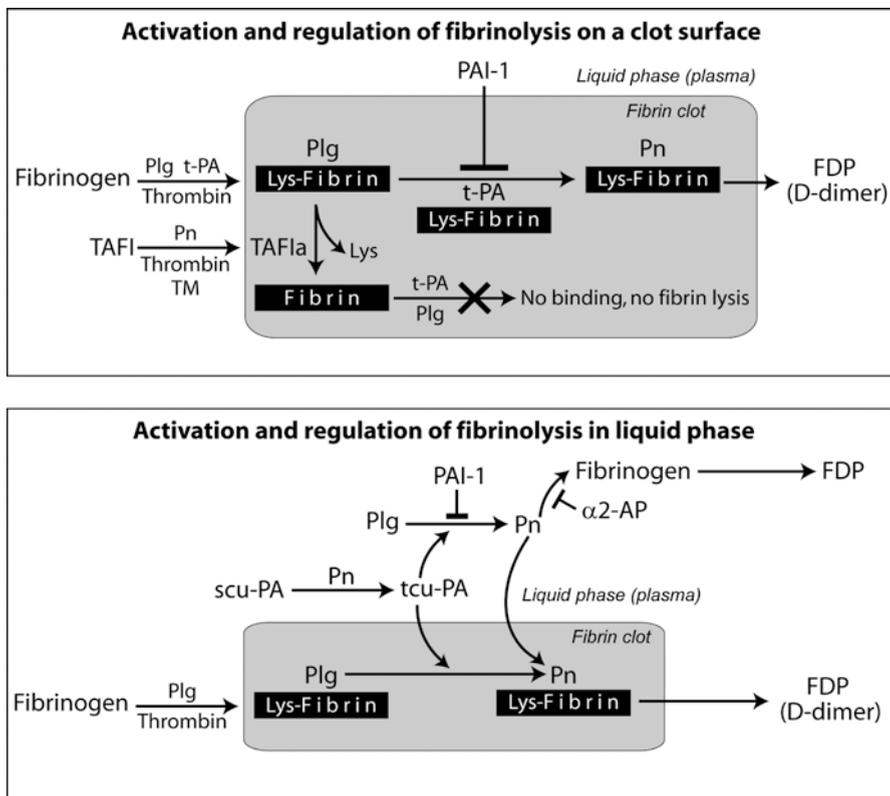
Fibrin sealants formed by mixing fibrinogen and thrombin are widely used to stop bleeding (Corral et al. 2016). Importantly, various commercially available fibrin sealants have diverse mechanical properties (Hickerson et al. 2011) and glues with distinct structure and stiffness had different hemostatic efficacy (Fortelny et al. 2011).

## 13.6 Lytic Stability of Fibrin

### 13.6.1 Molecular Mechanisms of Fibrinolysis

After a clot has formed *in vivo* and fulfilled its hemostatic function, it is normally dissolved by the fibrinolytic system, to restore the impaired blood flow (Weisel and Litvinov 2008, 2014; Longstaff and Kolev 2015). The main enzyme involved is plasmin (Pn), a serine protease derived from its inactive precursor, plasminogen (Plg), through the action of activators. Pn also cleaves several other substrates, including extracellular matrix proteins, and activates some proteases and growth factors. So, in addition to fibrinolysis, Plg and Pn are involved in several other physiological and pathophysiological processes, such as wound healing, inflammation, cell migration, angiogenesis, embryogenesis, ovulation, tumor growth and metastasis, and atherosclerosis.

There is a complex system of biochemical reactions comprising fibrinolysis and its regulatory mechanisms (Fig. 13.11). Fibrinolysis occurs first by the conversion of Plg to Pn by a Plg activator, primarily on the fibrin surface, and then by the degradation of fibrin by Pn. Any Pn that dissociates from fibrin is rapidly inactivated by inhibitors. The activators of Plg are the serine proteases tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator and bacterial proteins that acquire proteolytic activity after the interaction with human Plg or Pn, streptokinase and staphylokinase. t-PA and staphylokinase are fibrin-selective, remaining bound to fibrin and protected from rapid inhibition, while streptokinase and two-chain urokinase-type plasminogen activator are non-fibrin-selective enzymes, activating both Plg in the circulating blood and fibrin-bound Plg. Attachment of Plg and t-PA to fibrin is mediated by the C-terminal lysine residues of fibrin and the specific lysine-binding motifs on the Plg and t-PA molecules. Because Pn cleaves at lysine



**Fig. 13.11** Schematic diagram of fibrinolysis on the fibrin clot surface and in the liquid phase. Schematic representation of the major reactions of fibrinolysis and their regulation on a fibrin clot surface and in the surrounding plasma milieu. The *grey* highlighted area represents a fibrin clot surface (solid phase) surrounded by the blood plasma (liquid phase). *Black arrows* show the biochemical conversions involving proteolytic cleavage. *T-like symbols* indicate inhibitory effects. Abbreviations and functions of molecules: *Lys-Fibrin* C-terminal lysine residues on fibrin to which Plg and t-PA bind selectively, *Plg* plasminogen, bound to the C-terminal lysine residues on fibrin and free in plasma, *Pn* plasmin, formed on fibrin (by the action of t-PA) and in plasma (by the action of t-PA) from Plg. Pn cleaves fibrin and fibrinogen, activates scu-PA and TAFI, *t-PA* tissue-type Plg activator, fibrin-selective Plg activator, bound to fibrin via the C-terminal lysine residues on fibrin, *scu-PA* single-chain urokinase-type Plg activator (inactive), *tcu-PA* two-chain u-PA (active), non-fibrin-selective Plg activator, *PAI-1* plasminogen activator inhibitor-1, blocks both t-PA and t-PA to fibrin, *TAFIa* thrombin-activatable fibrinolysis inhibitor (enzymatically active form) that splits off the C-terminal lysine residues from fibrin, thus preventing binding of Plg and t-PA to fibrin, *α<sub>2</sub>-AP* α<sub>2</sub>-antiplasmin, direct Pn inhibitor, forms circulating Pn-α<sub>2</sub>-AP complexes, *FDP* fibrin(ogen) degradation products, resulting from cleavage of fibrin or fibrinogen by Pn, *D-dimer* a proteolytic fragment (degradation product) that is formed by Pn only from crosslinked fibrin

residues, the new C-terminal lysines provide additional binding sites for Plg and t-PA, as a positive feedback mechanism. The number of t-PA and Plg binding sites on fibrin and the rate of lysis can be increased by ultrasound-induced perturbation of fibrin clots, which may be of practical importance for thrombolytic therapy (Chernysh et al. 2015).

Enzymatic lysis of fibrin results in formation of soluble degradation products. Basically, the fibrin degradation products generated by plasmin are very similar to the proteolytic fragments of fibrinogen, namely intermediate fragments X and Y and the terminal fragments D and E, with the difference based on the presence or absence of fibrinopeptides (Marder and Budzynski 1975). What makes fibrin degradation products formed *in vivo* different from those of fibrinogen is the non-covalent interactions and covalent Factor XIIIa-mediated crosslinking of fibrin, which results in formation of various soluble oligomeric structures (Veklich et al. 1998; Horan and Francis 2001). The smallest crosslinked fibrin degradation product is D-dimer formed of two D regions of the adjacent fibrin molecules connected by a  $\gamma$ - $\gamma$  bond. Because the only source of D-dimer is crosslinked fibrin, a high level of D-dimer-containing products in blood is widely used by clinicians as a laboratory sign of intravascular fibrin deposition occurring during local thrombosis or disseminated intravascular coagulation (Bates 2012). Fibrin degradation products comprise fibrin(ogen) fragments with incomplete sets of binding sites that competitively inhibit fibrin polymerization and can result in formation of large oligomeric structures containing non-polymerized fibrin molecules named “soluble fibrin complexes” or “soluble fibrin”.

### 13.6.2 Modulators of Fibrinolysis

There are a number of biochemical reactions that moderate the activity of the profibrinolytic components, such as Plg activator inhibitor-1, a potent inhibitor of t-PA and u-PA, and  $\alpha_2$ -antiplasmin, which directly inhibits Pn (Fig. 13.11). Thrombin-activatable fibrinolysis inhibitor cleaves C-terminal lysine residues from partially degraded fibrin and thus inhibits fibrinolysis by preventing the lysine-dependent binding of Plg to fibrin. Lipoprotein(a) has a structural homology with Plg and, therefore can compete with Plg for binding to lysine residues and impair fibrinolysis (Angles-Cano et al. 2001).

The effectiveness of fibrinolysis results from the combination of regulated enzymatic activity and the physical characteristics of the fibrin scaffold, such as the density of fibers and branch points, pore size, and fiber diameter. In general, the rate of lysis appears to be faster for clots made up of thicker fibers than for clots made up of thinner fibers, but also depends on other biophysical properties of the clot (Weisel 2007). In addition, platelet aggregation and clot retraction have dramatic effects on fibrinolysis (Collet et al. 2002). Stretching also affects the rate of lysis of clots (Varju et al. 2011).

### 13.6.3 *Internal and External Fibrinolysis*

A variety of systems have been used to study fibrinolysis experimentally, and each has strengths and weaknesses and is suited to the investigation of different aspects of lysis. With each of these systems, there are often several biochemical or structural methods that can be used to quantify rates of lysis.

The process that mimics the physiological process of fibrinolysis has been called internal or intrinsic lysis. To simulate this process *in vitro*, either t-PA mixed with plasma or t-PA and Plg mixed with fibrinogen have been clotted, so that the clot is formed and then dissolved as the t-PA activates Plg to Pn on the fibrin surface. The process of lysis has been followed by measurement of the decrease of turbidity or by confocal or other light microscopy or biochemically by the appearance of lysis products (Gabriel et al. 1992; Collet et al. 2003).

The process that mimics clinical thrombolysis is called external or extrinsic fibrinolysis. For therapeutic thrombolysis, t-PA is introduced into the vasculature, so that it circulates and binds to thrombi, activating Plg to Pn on the fibrin surface. *In vitro*, t-PA can be introduced at the edge of a pre-formed clot in a chamber, commonly made of a light microscope slide and cover slip, so that lysis can be observed either by eye or by light microscopy (Sakharov and Rijken 1995; Collet et al. 2000). In the external lysis system just described, the t-PA enters the clot only by diffusion. However, in thrombolysis the t-PA is delivered by flowing blood, so there usually is permeation or perfusion into the thrombus. Thus, a variation of external lysis is to allow the t-PA to permeate into the clot. The results can be quantified by the measurement of digestion products released or by observation of changes in clot structure as a function of time.

## 13.7 Conclusions

During the past 10 years, all of these different facets of research have contributed to the vigorous flowering of new information about fibrinogen and fibrin. It is now well established that fibrin is essential for hemostasis and is a major factor in thrombosis, inflammation and infection. Many of the molecular mechanisms of the biological processes of hemostasis and how they go awry in thrombosis have been determined. The binding of fibrinogen to the integrin  $\alpha\text{IIb}\beta\text{3}$  and its role in platelet aggregation continues to represent a model system to study cell adhesion. In addition, fibrin has become increasingly used as a versatile and unique biomaterial.

In spite of the breadth and depth of research on fibrin(ogen) in the past 10 years, many mysteries remain. For example, molecular mechanisms of lateral aggregation and branching in fibrin polymerization are largely unknown. Since the preponderance of research on fibrinogen and fibrin has been on mammalian systems, much remains unknown about their roles in other animals. Many of the binding partners of fibrin(ogen) have been identified but their functions are mostly unknown. Basic

mechanisms of the catabolism of fibrinogen remain to be discovered. The burgeoning field of fibrin mechanics will continue to thrive. Much remains to be learned about biological roles of fibrinogen beyond hemostasis. In the future, research will increasingly investigate major aspects of the role of fibrin(ogen) in more complex biological processes, such as wound healing and inflammation. The diverse array of powerful genetic and synthetic tools in various model systems, together with new super-resolution, single-molecule and single-cell imaging and manipulation methods, will drive this research. One thing is certain, fibrinogen and fibrin will continue to surprise us in the future, as they have in the past.

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