In Vitro Assembly of the Component Chains of Fibrinogen Requires Endoplasmic Reticulum Factors*

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Human fibrinogen (340 kDa) is a dimer, with each identical half-molecule composed of three different polypeptides (Aα, 66 kDa; Bβ, 55 kDa; and γ, 48 kDa). To understand the mechanisms of chain assembly, a coupled in vitro transcription-translation system capable of assembling fibrinogen chains was developed. Fibrinogen chain assembly was assayed in an expression system coupled to rabbit reticulocyte lysate in the presence or absence of dog pancreas microsomal membranes. Fibrinogen chain assembly required microsomal membranes and oxidized glutathione. Co-expression of two of the chains, Bβ and γ or Aα and γ, yielded free chains and two-chain complexes. Unlike combinations of Aα with γ and Bβ with γ, co-expression of Aα and Bβ did not form a single two-chain complex but produced a mixture of two-chain complexes. Co-expression of all three chains yielded free chains, two-chain complexes, and higher molecular weight complexes that corresponded to a half-molecule and to fully formed fibrinogen. Upon treatment of this mixture with thrombin and factor XIIIa, a γγ dimer, similar to that obtained from cross-linked human fibrin, was produced, indicating that properly folded fibrinogen was formed in vitro. Molecular chaperones may participate in fibrinogen assembly, since antibodies to resident proteins of the endoplasmic reticulum (BiP, Hsp90, protein disulfide isomerase, and calnexin) co-precipitated the chaperones together with nascent fibrinogen chains and complexes.

Human fibrinogen is a dimer with each half-molecule containing three nonidentical polypeptide chains (Aα, Bβ, and γ). Two of the chains, Bβ and γ, are glycoproteins. Fibrinogen is an elongated, trinodal molecule composed of a central domain (E domain), which contains the NH2 termini of the six chains, and two terminal D domains, formed by the carboxyl-terminal globular regions of Bβ and γ chains and a segment of the Aα chain. The central E domain and the terminal D domains are connected by a “coiled-coil” α-helical region composed of the three chains. The coiled region is flanked at both ends by a set of interchain “disulfide rings.” In the central E domain the 2 half-molecules are joined at their NH2-terminal regions by three symmetrical disulfide bonds between adjacent Aα Cys-28 and γ Cys-8 and γ Cys-9 residues (for reviews see Refs. 1–5). Site-directed mutagenesis, changing NH2-terminal cysteine residues to serine, also indicates that Aα Cys-36 of one half-molecule is disulfide-linked to Bβ Cys-65 of the other half-molecule (6, 7). Fibrinogen has a total of 29 inter- and intra-chain disulfide bonds with no free cysteine residues (8–13). In addition, human plasma contains a small amount of fibrinogen, which has an extended Aα chain with a COOH-terminal globular domain similar to that of the Bβ and γ chains. (14, 15).

Chain assembly into six-chain fibrinogen occurs in the endoplasmic reticulum (ER) of hepatocytes (16). As with other multichain proteins, proper folding takes place in the ER before the nascent protein progresses to the Golgi complex, where further processing occurs, and before it is secreted. For most proteins initial assembly and folding is facilitated by transient interactions with a number of molecular chaperones that reside in the ER (for reviews see Refs. 17–19).

Pulse-chase studies with Hep G2 cells demonstrated the step-wise assembly of fibrinogen chains, progressing from two-chain complexes to three-chain half-molecules, which are finally coupled together to form six-chain fibrinogen (20, 21). Several recombinant fibrinogen systems have been described that are capable of assembling and secreting fibrinogen (22–25). The intracellular complexes found in some of these cells are similar to those in Hep G2 cells, suggesting similar paths of assembly. Early pulse-chase studies with Hep G2 cells showed accumulation of an Aαγ complex and postulated that AαBβ and Bβγ are also precursors in the formation of half-molecules and six-chain fibrinogen (21, 26). The accumulation of precursor and intermediate fibrinogen complexes under steady-state conditions have been characterized in Hep G2 cells and in transfected COS and baby hamster kidney cells. In these cells Aαγ, Bβγ, half-molecules, and six-chain fibrinogen were identified, but AαBβ complexes were not detected (27). AαBβ complexes were also not found in transiently transfected COS cells (28). However, all of the studies indicate a stepwise assembly, from single chains to two-chain complexes, addition of a third chain to form the half-molecule, and finally dimerization to form the final six-chain complex. Studies with transfected cells, using deletion and substitution mutants of the fibrinogen chains, indicate that formation of the coiled-coil structure and its disulfide rings play important roles in dimer formation (7).

To further understand the mechanisms of fibrinogen assembly, an in vitro system that couples transcription, translation, and events that normally occur in the ER has been developed.

EXPERIMENTAL PROCEDURES

Materials—TNT, T7-coupled reticulocyte lysate system, TNT RNA polymerase (T7), Rnasin ribonuclease inhibitor, and canine pancreatic microsomal membranes were purchased from Promega (Madison, WI). L-[35S]methionine (1000 Ci/mmol) was obtained from Amersham Corp., and restriction enzymes, Klenow fragment, and calf intestinal phospho-

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† The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; kb, kilobases; COS, monkey kidney fibroblast cells; BiP, immunoglobulin-binding protein; GRP, glucose-regulated stress protein.
termed pYES2A at the 3' end of T7 promoter at the appropriate restriction sites and right orientations. Two- and three-chain combinations were inserted in tandem, each under the control of a T7 promoter. A diagram is shown at the top.

To make the two-chain construct pYES2A\textsubscript{g}, a 2.5-kb fragment (GalT7\textsubscript{a}) was released from pYES2\textsubscript{a} by BamHI and partial SpeI digestion. The 2.5-kb fragment was ligated to SpeI-cut, dephosphorylated pYES2B\textsubscript{b} plasmid followed by a fill-in reaction and blunt end ligation. To construct pYES2A\textsubscript{g}, the GalT7\textsubscript{a} fragment was ligated to BamHI-cut, dephosphorylated pYES2\textsubscript{g} plasmid that had been treated with BamHI, followed by a fill-in reaction and blunt end ligation. To obtain pYES2B\textsubscript{b}\gamma, a GalT7\textsubscript{γ} (2.0-kb) fragment was released from pYES2\textsubscript{γ} plasmid by SpeI and ligated to SpeI-cut, dephosphorylated pYES2B\textsubscript{b} plasmid.

To obtain the construct with all three cDNAs (pYES2A\textsubscript{g}, B\textsubscript{b}\gamma) the GalT7\textsubscript{a},GalT7\textsubscript{a} fragment (4.5 kb) was released from pYES2A\textsubscript{g},B\textsubscript{b}\gamma by BamHI and partial SpeI treatment and was ligated to SpeI-cut, dephosphorylated pYES2B\textsubscript{b}\gamma plasmid, followed by a fill-in reaction and blunt end ligation. The orientation of the cDNAs in the above constructs was determined by treatment with the appropriate restriction enzymes. The procedures for elution of DNA fragments from agarose gel, dephosphorylation of plasmids by calf intestinal phosphatase, the fill-in reaction by Klenow fragment, and ligation were performed by standard procedures (29).

Cell-free Transcription and Translation of Fibrinogen Chains—Transcriptions and translations were performed using the TNT\textsuperscript{T7} transcription system coupled with a rabbit reticulocyte system according to the manufacturer's directions. 

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Fig. 1. Expression vectors containing fibrinogen chain cDNAs. Full-length cDNAs were inserted into multiple cloning sites at the 3' end of T7 promoter at the appropriate restriction sites and right orientations. Two- and three-chain combinations were inserted in tandem, each under the control of a T7 promoter. A diagram is shown at the top.
RESULTS

Synthesis and Processing of Fibrinogen Chains—In vitro expression of polypeptides in the presence of stripped dog pancreas microsomes leads to initial proteolytic processing, translocation of the nascent polypeptides into the lumen of microsomes, and glycosylation of the Bβ and γ chains. The addition of microsomal membranes to the translation mixture containing pYES2Aα, pYES2Bβ, and pYES2γ resulted in a mobility shift as determined by SDS-PAGE of Bβ and γ chains, which are glycoproteins, but not of Aα (Fig. 2). Similarly, when combinations of two chains or all three chains were expressed, the Bβ and γ chains, but not Aα, had slower mobilities on SDS-PAGE in the presence of microsomal membrane (Fig. 2). Multiple smaller fragments of all fibrinogen chains were observed. These small polypeptides are probably due to incomplete translation products and also to degradation.

To determine whether N-glycosylation occurred, the translation products of pYES2Aα, Bβγ with microsomal membranes were immunoprecipitated and treated with endoglycosidase H. Endoglycosidase H treatment did not affect the mobility of the chains expressed in the absence of microsomal membranes but increased the mobilities of Bβ and γ chains, translated together with microsomal membranes. This indicates that the Bβ and γ chains were N-glycosylated and contain mannose-rich oligosaccharides. The electrophoretic migration of Aα chain, which normally is not N-glycosylated, was not affected by treatment with endoglycosidase H (data not shown).

Other Methods—Endoglycosidase-H treatment of translated products, thrombin treatment, and cross-linking with factor XIIIa were performed as described previously (28).

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Fig. 2. Transcription-coupled translation of fibrinogen chain cDNAs. Single fibrinogen chains, combinations of two chains, and all three fibrinogen chains were expressed in the presence (+) or absence (−) of canine pancreatic microsomal membranes and separated by 7.5% SDS-PAGE under reducing conditions. An autoradiogram is shown. Aα was expressed from pYES2Aα, Bβ from pYES2Bβ; γ from pYES2γ; AαBβ from pYES2Aα,Bβ; Aαγ from pYES2Aα,γ; Bβγ from pYES2Bβ,γ; and AαBβγ from pYES2Aα,Bβ,γ. Molecular size markers are indicated on the left, and the locations of Aα, Bβ, and γ chains are shown on both sides of the autoradiogram. The fibrinogen chains expressed are shown at the top.

Chain Assembly Requires Oxidized Glutathione—Oxidized glutathione, added to cell-free systems, has been shown to result in the correct disulfide-bonded assembly of biologically active proteins (30–33). To determine whether oxidized glutathione was necessary for fibrinogen chain assembly, pYES2Aα,Bβ,γ was transcribed and translated in the presence of microsomal membranes and varying concentrations of GSSG. Analysis of nascent proteins, in nonreduced SDS-PAGE showed that in the absence of GSSG free chains and high molecular weight aggregate were the principal products. In the presence of 1 mM GSSG two-chain complexes (Aα-γ and Bβ-γ) and some higher molecular weight products were formed. With increased concentrations of GSSG (2–15 mM), more of these assembled fibrinogen complexes appeared. The optimum concentration of GSSG was about 5–10 mM.

Kinetic studies showed that at the earliest time measured (15 min) mostly free Aα, Bβ, and γ chains were present, with larger amounts of γ chains. Intermediate two- and three-chain complexes and material, similar in size to fibrinogen, were detected at 30 min and continued to accumulate until 120 min of incubation. Scanning densitometric analyses showed that the percentage of radioactivity in free chains decreased with time and that the radioactivity in fibrinogen and its intermediates complexes increased. At the end of 120 min of incubation, approximately 20% of the total protein radioactivity was accounted for in a location where six-chain fibrinogen migrates and 15–20% was in intermediate two-chain and three-chain complexes (Fig. 3).

Assembly of Two- and Three-chain Complexes Requires Microsomal Membranes—Two- and three-chain complex formation required that the chains be translated in the presence of microsomal membranes. In the absence of microsomal membranes only free chains, large molecular weight aggregates, and proteins that migrated as a streak on SDS-PAGE were noted (data not shown). In the presence of microsomal membranes, co-translation of Aα and γ and of Bβ and γ led to the assembly of discrete bands that migrated as expected for two-chain complexes (Fig. 4, B and C). However, co-translation of
Aα and Bβ did not yield a distinct two-chain product, although a darker diffuse area was noted in the region where two-chain complexes are expected to migrate (see Fig. 4A). Co-translation of all three chains, in the absence of microsomal membranes, led to the formation of a high molecular weight aggregate and free chains. In the presence of microsomal membranes, in addition to free chains, two bands were observed in the location where two-chain complexes migrate. Higher molecular weight products, corresponding to half-molecules (three-chain complexes) and to six-chain fibrinogen were also formed (Fig. 4D).

Two-dimensional SDS-PAGE Analysis—To characterize the complexes formed when different chains are co-translated in the presence of microsomal membranes, the translation products, isolated by immunoprecipitation with polyclonal antibody to fibrinogen, were analyzed by two-dimensional SDS-PAGE first in nonreduced conditions and then in reducing conditions.

Co-expression of Aα and Bβ yielded, in nonreducing conditions, aggregated material at the top of the gel, a diffuse band at the region where two-chain complexes are expected to migrate, and free chains. Upon reduction and electrophoresis in a second dimension, both the high molecular weight aggregate and the area that may contain two-chain complexes yielded Aα and Bβ chains. Greater amounts of Aα chain than Bβ chains were present in these areas. The free chains, as expected, gave single bands (Fig. 4A).

Co-expression of Aα and γ yielded two bands that, because of their electrophoretic migration in the first dimension, are putative two-chain complexes. One higher molecular weight band was composed of Aα and γ chains, and there was indication of a γγ dimer, which migrated just below the Aα-γ complex. Free Aα and γ chains were also present (Fig. 4B).

Co-expression of Bβ and γ chains gave a single two-chain complex and free chains. There were many more free γ than free Bβ chains. There were few, if any, higher molecular weight complexes formed. Upon reduction, in the second dimension, the two-chain complex yielded Bβ and γ chains (Fig. 4C).

Co-translation of all three chains showed the presence of several complexes (Fig. 4D). In the areas where six-chain fibrinogen and the half-molecules are expected to migrate, Aα, Bβ, and γ chains were obtained. There was greater recovery of Aα and Bβ than of γ chains. Similarly, in the area where two-chain complexes migrate, Aα and γ chains were detected in one area and Bβ and γ from another area. In all cases there was much lower recovery of γ chains in the second dimension. The reasons for this are not clear, although partly it is due to lower methionine content and, therefore, less radioactivity, in γ as compared with Aα and Bβ chains. Fewer γ than Aα and Bβ chains were also noted in the regions where fibrinogen, half-molecules, and the two-chain complexes migrate.

Characterization of AαBβγ Complexes with Chain-specific Antibodies—To further characterize the complex formed when Aα and Bβ are co-translated in the presence of microsomal membranes, the translation products, isolated by immunoprecipitation with polyclonal antibody to fibrinogen, were analyzed by two-dimensional SDS-PAGE first in nonreduced conditions and then in reducing conditions.

Co-expression of Aα and Bβ yielded, in nonreducing conditions, aggregated material at the top of the gel, a diffuse band at the region where two-chain complexes are expected to migrate, and free chains. Upon reduction and electrophoresis in a second dimension, both the high molecular weight aggregate and the area that may contain two-chain complexes yielded Aα and Bβ chains. Greater amounts of Aα chain than Bβ chains were present in these areas. The free chains, as expected, gave single bands (Fig. 4A).

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Co-expression of Bβ and γ chains gave a single two-chain complex and free chains. There were many more free γ than free Bβ chains. There were few, if any, higher molecular weight complexes formed. Upon reduction, in the second dimension, the two-chain complex yielded Bβ and γ chains (Fig. 4C).

Co-translation of all three chains showed the presence of several complexes (Fig. 4D). In the areas where six-chain fibrinogen and the half-molecules are expected to migrate, Aα, Bβ, and γ chains were obtained. There was greater recovery of Aα and Bβ than of γ chains. Similarly, in the area where two-chain complexes migrate, Aα and γ chains were detected in one area and Bβ and γ from another area. In all cases there was much lower recovery of γ chains in the second dimension. The reasons for this are not clear, although partly it is due to lower methionine content and, therefore, less radioactivity, in γ as compared with Aα and Bβ chains. Fewer γ than Aα and Bβ chains were also noted in the regions where fibrinogen, half-molecules, and the two-chain complexes migrate.
membranes, the radioactive products were isolated with antibodies specific to Aα and Bβ chains. In nonreducing conditions (first dimension) the pattern obtained with the chain-specific antibodies was slightly different from that obtained with the polyclonal antibody to fibrinogen. In addition to the free chains and the diffuse streaking in the high molecular weight region, a distinct band was obtained just above the 200-kDa marker. Upon reduction (second dimension), this band, isolated with antibody to Bβ, yielded mostly Aα chain with a trace of radioactive material in the region where Bβ migrates. The “diffuse” streak between the 200- and 100-kDa markers was composed mostly of Aα chains. Free Aα chain, and lower molecular weight bands, which could be degradation products or incomplete Aα chains, were also detected (Fig. 5A). Isolation with anti-Bβ showed that the radioactive product, which migrated just above the 200-kDa marker, contained mostly Bβ but also contained a trace of Aα and proteins with lower molecular weights than Bβ. The diffuse streak between the 200- and 100-kDa markers was mostly composed of Bβ (Fig. 5B). Free Bβ chains and proteins of lower size than Bβ were also isolated.

Taken together, these results indicate that co-expression of Aα and Bβ leads mainly to the formation of high molecular weight homocomplexes. Very few heterocomplexes of Aα and Bβ are formed, although there is an indication that some Aα and Bβ chains are assembled together, since anti-Bβ isolates small amounts of Aα (Fig. 5B) and anti-Aα isolates small amounts of Bβ (Fig. 5A).

**Co-precipitation of Nascent Fibrinogen and Molecular Chaperones**—The three fibrinogen chains were co-expressed in the presence of microsomal membranes and GSSG, and the radioactive proteins formed were treated with nonimmune rabbit serum as a control or with rabbit antiserum to fibrinogen, GRP78 (BiP), protein disulfide isomerase, or calnexin or with mouse monoclonal antibody to GRP90. The radioactive proteins, present in the immunoprecipitate, were separated by one- or two-dimensional SDS-PAGE and detected by autoradiography.

No radioactive proteins were isolated with nonimmune rabbit serum. The other antibodies precipitated fewer radioactive proteins than anti-fibrinogen, but the proteins isolated with the antibodies to the different chaperones had the same electrophoretic properties as those isolated by anti-fibrinogen. The antibodies to chaperones isolated less radioactive proteins than anti-fibrinogen. Anti-calnexin isolated 15%, and the other antibodies isolated 25–30% as compared with anti-fibrinogen, (Fig. 6A).

Two-dimensional SDS-PAGE was performed to identify the precipitated radioactive proteins. All of the antibodies to the molecular chaperones isolated free Aα and γ chains (Fig. 6, C, D, E, and F). Very little free Bβ chain was present, even in the immunoprecipitate isolated with anti-fibrinogen. An Aα-γ complex (which migrated below fibrinogen) was also isolated by all of the antibodies tested. The half-molecule, which is detected upon isolation with anti-fibrinogen (it migrated below fibrinogen) (Fig. 6B) was only clearly identified with anti-GRP90 (Fig. 6D) and anti-protein disulfide isomerase (Fig. 6E). Very few half-molecules were detected in the precipitate obtained with anti-BiP (Fig. 6C) and anti-calnexin (Fig. 6F). Fibrinogen, which migrates at the top of the band and is identified by yielding the three component chains upon reduction (Fig. 6B), was isolated by anti-BiP (Fig. 6C), anti-GRP90 (Fig. 6D), and anti-protein disulfide isomerase (Fig. 6E). Although anti-calnexin co-isolated, on the first dimension, a protein that migrated similarly to fibrinogen, this compound contained very little of the three component chains of fibrinogen (Fig. 6F). The high molecular weight proteins isolated by anti-calnexin could be aggregated forms of fibrinogen polypeptides.

**Factor XIIIa-catalyzed Cross-linking of in Vitro Fibrinogen**—A functional test for fibrinogen is its ability to undergo thrombin-induced polymerization and factor XIIIa-catalyzed cross-linking. To determine whether the in vitro assembled fibrinogen met this criterion, the total radioactive proteins, obtained by co-expression of all three chains in the presence of GSSG and microsomal membranes were treated with thrombin and factor XIIIa. Plasma fibrinogen treated in this manner yields high molecular weight cross-linked Aα chains, γγ dimers, and unreacted Bβ chains. In vitro translated fibrinogen chains gave γγ dimer, indicating the presence of some properly assembled fibrinogen, but a substantial amount of free Aα and γ chains remained, indicating that a number of translated chains were not assembled into six-chain fibrinogen capable of undergoing polymerization and cross-linking (data not shown).

**DISCUSSION**

In hepatocytes, assembly of the component chains of fibrinogen into the final six-chain molecule occurs in a stepwise fashion. First, single chains are linked to form two-chain complexes, and later these duplexes acquire a third chain to form...
three-chain half-molecules. In the final step two half-molecules are joined to form the six-chain fibrinogen molecule (20, 21, 26, 27). In the in vitro system this cellular assembly process appears to be duplicated, since similar precursor and intermediate products, are formed. Assembly of the chains requires GSSG and microsomal vesicles, derived from the ER. GSSG is needed for the in vitro formation of disulfide bonds in other proteins (30–33). The ER, in contrast to the cytoplasm, maintains an oxidizing environment, with ratios of GSSG and GSH primarily contributing to its redox condition (34). Although this oxidizing environment ensures disulfide formation of most proteins, the process is facilitated by protein disulfide isomerase, an abundant ER enzyme that catalyzes thiol/disulfide interactions and promotes disulfide bond formation and isomerization (35, 36). Therefore, it is not surprising that nascent fibrinogen, which contains 29 disulfide bonds, may interact transiently with protein disulfide isomerase and is co-precipitated with an antibody specific for this chaperone.

Fibrinogen chain assembly also requires microsomal membranes, and in their absence the expressed chains do not form recognizable chain complexes. The ER contains, in addition to protein disulfide isomerase, a number of resident proteins that function to recognize nascent proteins, prevent incorrect association or folding, and assist in proper folding and stabilization. These early co- and post-translational steps are required before the nascent proteins exit the ER and travel to the Golgi, where further processing occurs (17–19). In keeping with this view, antibodies to several ER chaperones co-precipitated free chains, intermediate fibrinogen complexes, and nascent fibrinogen. Previously it was shown, in both hepatocytes and recombinant systems, that BiP, a resident protein of the ER, is associated with fibrinogen (37). The association of nascent fibrinogen and its precursors with different chaperones varied. In all cases free Aα and γ chains and Aαλγ duplex, appeared to be complexed to all the chaperones. On the other hand, half-molecules were not detected with BiP or calnexin, and six-chain fibrinogen was not noted together with calnexin. Failure to detect these associations does not necessarily mean that the nascent fibrinogen chains are not complexed to the chaperones, but it may reflect low recovery, perhaps due to a loose transient association, which could be disrupted by the experimental procedures employed. It is interesting to note that calnexin, which is an integral membrane protein of the ER and is thought to interact with nascent proteins during initial N-glycosylation.
In vitro fibrogenin chain assembly resembles that occurring in hepatocytes and in recombinant systems. In all cases more free Aα and γ chains than Bβ chains accumulate in the system. This could be due to rapid assembly of Bβ chains into larger complexes, coupled with degradation of free Bβ chains. Earlier studies with Hep G2 cells indicated that Bβ is tagged by BiP and is degraded in the ER (37). In the in vitro system there are also noticeable accumulations of several intermediates, including Aαγ and Bβγ duplexes, and half-molecules. In the in vitro system, as in steady-state labeled Hep G2 cells (27), there was little Aα-Bβ duplex formation. Co-expression of only Aα and Bβ chains led to the formation of Aα and Bβ homopolymers with only minor amounts of Aα-Bβ duplex assembly. Thus, both in vitro and in Hep G2 cells (20, 21, 27) fibrogenin chain assembly appears to commence with the formation of two-chain complexes, primarily Aαγ and Bβγ, followed by the addition of a third chain to form bi-molecules, which dimerize to form the six-chain final product. Covalent disulfide bond interactions, especially those that flank the coiled-coil region, are important in stabilizing the complexes and allowing chain assembly (7). Final linkage of the two half-molecules involves both nonglycosylated and glycosylated proteins but that nonglycosylated domains may also be recognized (39, 42–46).

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