The Assembly of Human Fibrinogen

THE ROLE OF THE AMINO-TERMINAL AND COILED-COIL REGIONS OF THE THREE CHAINS IN THE FORMATION OF THE αγ AND βγ HETERODIMERS AND αβγ HALF-MOLECULES*

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Fibrinogen is a plasma protein consisting of six polypeptide chains which are linked by disulfide bonds. During protein synthesis, assembly of the molecule proceeds through the formation of αγ and βγ heterodimers followed by the generation of αβγ half-molecules and dimerizing to generate the mature six-chain molecule. In the present study, sequences required for the formation of the αγ and βγ heterodimers were examined in stably transfected baby hamster kidney cells expressing combinations of normal as well as modified polypeptide chains. Deletion of the amino terminus and the proximal first half of the coiled-coil region of the three fibrinogen chains had little or no effect on heterodimer and half-molecule formation. These deletions, however, did prevent half-molecules from forming the six-chain molecule. Deletion of the distal second half of the coiled-coil region of each chain completely prevented the assembly process. Point mutations in the second half of the coiled-coil region also indicated that hydrophilic residues that form ion pairs between interacting chains were not critical in the formation of the heterodimeric complexes. These results suggest that the initial formation of the αγ and βγ complexes depends primarily on hydrophobic interactions of amino acids located in the second half of the coiled-coil region of the molecule. These interactions occur in the rough endoplasmic reticulum in the presence of various chaperones such as BiP.

Human fibrinogen is a plasma glycoprotein that participates in the final phase of blood coagulation. It is composed of two sets of three polypeptides, designated α, β, and γ with molecular masses of 66, 52, and 46 kDa, respectively (McKee et al., 1966). The six chains, (αβγ)2, are linked by 29 interchain and intrachain disulfide bonds to form a trinodular structure (340 kDa) as seen in the electron microscope (Hall and Slayter, 1959). The central nodule contains the amino termini of all six polypeptide chains, whereas the two outer nodules contain the carboxyl termini of the β and γ chains (Norton and Slayter, 1981; Weisel et al., 1985). The outer nodules can be subdivided into two closely positioned globular domains formed by the independent folding of the β and γ chains (Rao et al., 1991). The carboxyl terminus of the α chain folds into a compact domain that folds back toward the middle of the molecule where it interacts with the central nodule (Veklich et al., 1993). The three nodules of fibrinogen are connected by coiled-coils composed of two triple-stranded helices involving 110 or 111 amino acids (Doolittle et al., 1978).

Several pathways of fibrinogen assembly have been proposed (Roy et al., 1991; Hartwig and Danshiefsky, 1991). Studies from our laboratory employing stably transfected baby hamster kidney (BHK) cells showed that the initial assembly of fibrinogen involves the formation of αγ and βγ heterodimers, but not αβ heterodimers (Huang et al., 1993). The addition of a third chain leads to the formation of half-molecules (αβγ), which then dimerize to form the mature six-chain fibrinogen, (αβγ)2, that circulates in blood.

The three genes coding for the α, β, and γ chains of human fibrinogen are located on chromosome 4 in about 50 kilobase pairs of DNA (Henry et al., 1984). They are arranged in the order of γ, α, and β and contain a number of introns (four, seven, and nine introns, respectively) that may serve in part to delineate functional domains in the protein (Chung et al., 1990; Kant et al., 1985). The three genes for human fibrinogen also contain a number of conserved introns, including one located in the middle of the coiled-coil domain in each of the chains (α103–104, β133–134, and γ76–77) (Medved, 1990). The location of this intron also coincides with plasmagin-sensitive sites in each of the three chains, and corresponds to a region in the triple-stranded helix that can accommodate a significant deviation from a perfect triple-stranded coil (Doolittle et al., 1978). It seems possible that the location of this intron may also serve to delineate sequences in the fibrinogen chains that are necessary for the assembly of the three chains.

In the present study, various combinations of chimeric fibrinogen chains (αβγ, αγγ, βγα, βαγ, γαβ, and γβα) were constructed using this intron position as the crossover point in order to assess the contribution of the amino terminus and the first half of the coiled-coil region of each chain in the assembly process.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, Taq DNA polymerase, and Sequenase were purchased from Promega, Boehringer Mannheim, and U. S. Biochemical Corp., respectively. Cell culture media were obtained from Life Technologies, Inc. and JRH Scientific, and fetal bovine serum was obtained from Hyclone. [35S]Met and [35S]Cys (1.1 Ci/mmol) were from Amerham. Protein A-Sepharose was obtained from Sigma, and polyclonal antibodies against human fibrinogen from Accurate Chemical.

Mutagenesis and Expression Plasmid Construction—cDNAs coding for point mutations, deletions, and chimeric fibrinogen chains were constructed by the polymerase chain reaction extension method of Higuchi et al. (1988) using chimeric or mutated primers and flanking primers. The plasmids pAZem229, pBZem229, and pGZem229 coding for the wild-type α, β, and γ chains of human fibrinogen (Huang et al., 1993) were used as templates. The polymerase chain reaction products were cloned into the EcoRI site of the expression vector pZem229R, which carried a copy of the cDNA coding for the selection marker dihydrofolate reductase (Mulvihill et al., 1988). The sequence of each

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† The abbreviations used are: BHK, baby hamster kidney; BiP, immunoglobulin heavy chain-binding protein; PAGE, polyacrylamide gel electrophoresis.
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**Fig. 1. Nomenclature and schematic representation of normal and chimeric fibrinogen chains.** A, normal fibrinogen chains: α, β, or γ chain is depicted by bars with different patterns. Vertical lines mark the positions of the proximal and distal disulfide rings. The set of conserved intron sites is indicated by the arrow. Substituted segments in chimeric constructs are labeled above the bars by the numbers of the first and last amino acid residues in the normal fibrinogen chain. B, chimeric fibrinogen chains: the chimeric fibrinogen chains are named according to the order of the segments from the respective chains, at the right. The abbreviations for them are listed at the left.

The construct was confirmed by dideoxy sequencing (Sanger et al., 1977).

**Cell Culture and DNA Transfection—**BHK 570 cells (ATCC #CRL1632) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, and antibiotics (penicillin, 50 U/ml; streptomycin, 50 μg/ml; and neomycin, 100 μg/ml). Cells were cultured to 80% confluence in 15-cm culture dishes and were transfected with 25 μg of each expression plasmid according to a modified calcium phosphate precipitation procedure (Graham and van der Eb, 1973). A final concentration of 1 μM methotrexate was then added to the culture medium 24 h post-transfection. Resistant colonies were clonally expanded into stably transfected cell lines. The copy number of transfected plasmids in some transfected cell lines was further amplified by exposure to and maintained in medium containing 20 μM methotrexate. The expression levels of fibrinogen chains in the transfected cell lines were assessed by Western blot analyses of cell extracts and culture media.

**Metabolic Labeling—**Transfected BHK cell lines expressing combinations of fibrinogen chains were plated onto 35-mm culture dishes. When the cells reached 90% confluence, the medium was replaced with Met and Cys-free Dulbecco’s Eagle’s medium containing 5% dialyzed fetal bovine serum. The cells were labeled for 18 h in the presence of 50 μCi/ml of each of [35S]Met and [35S]Cys. Cell lysates were prepared and subjected to SDS-PAGE as described previously (Huang et al., 1993).

**Immunoprecipitation and Electrophoresis—**Radioactively labeled intermediates of fibrinogen assembly from the cytosol and culture medium were bound to a polyclonal rabbit anti-human fibrinogen antibody and adsorbed onto Protein A-Sepharose as described previously (Huang et al., 1993). The intermediates were released from Protein A-Sepharose by boiling in 1.5 × SDS-PAGE sample buffer.

The composition of the intermediates was analyzed by two-dimensional SDS-PAGE, 7.5% polyacrylamide gel non-reduced in the first dimension. Gel strips were excised, reduced by 5% 2-mercaptoethanol, and electrophoresed in the second dimension in 10% polyacrylamide gels as described previously (Huang et al., 1993). The gels were impregnated with Amplify (Amersham), and dried. Radioactivity in the component fibrinogen chains was quantitated by a PhosphorImager (Molecular Dynamics) and the associated computer program Image Quant. The specific radioactivity for each component chain was determined by normalizing the radioactivity of the component chains to the total methionine and cysteine content in each chain. The dried gels were also exposed to x-ray films (XAR-5, Kodak) with intensifying screens at −70°C.

**RESULTS**

**Construction and Assembly of Chimeric Fibrinogen Chains—**Sequences important in the formation of the initial αγ/βγ complexes in the assembly process were studied by constructing various combinations of chimeric fibrinogen chains using common introns as a crossover point (Fig. 1). Studies were then performed in transfected BHK cells expressing a normal fibrinogen chain and a chimeric variant chain.

Coexpression of normal α chains with a chimeric β/γ chain led to the formation of an intracellular complex with an apparent mass of 152 kDa as shown by SDS-PAGE. Upon reduction by 2-mercaptoethanol, this complex gave rise to an α chain (63 kDa) and a chimeric β/γ chain (51 kDa) (Fig. 2A). These experiments showed the feasibility of using chimeric fibrinogen chains to study the assembly process. The electrophoretic mobility of the partially assembled complexes significantly deviated from linearity in one-dimensional SDS-PAGE as previously reported (Huang et al., 1993). The second-dimensional analysis following the reduction of disulfide bonds and quantitation of the radioactivity in the component chains was also employed to establish and confirm the composition of the complexes as described under “Experimental Procedures.”

When the β chain and the chimeric α/γ chain were coexpressed, a major complex with an apparent mass of 132 kDa was formed. It was readily resolved into a β and a chimeric α/γ chain following reduction (Fig. 2B). Furthermore, each chain was present in equimolar amounts (1:1.1) as shown by quantitation of the radioactive in the component chains. Similarly, coexpression of two chimeric chains, e.g., βα with α/γ, or α/β with β/γ chimeric chains, also led to the formation of heterodimeric intermediates (Fig. 2, C and D). These experiments indicated that one chimeric fibrinogen chain readily combines
with a normal fibrinogen chain, or with another chimeric fibrinogen chain to form αγ or βγ complexes. Furthermore, the synthesis and assembly process for these complexes appears to involve the chaperone BiP which is always co-precipitated with the fibrinogen chains (Fig. 2).

In contrast, coexpression of a γα chimeric chain with an αβ chimeric chain, or a normal α chain with a γβ chimeric chain led only to an accumulation of individual chains in the cells with no detectable heterodimer formation (Fig. 2, E and F). Similarly, coexpression of a βα chimeric chain with a γβ chimeric chain, or a normal β chain with a γα chimeric chain did not generate a heterodimer (data not shown). Subsequently, chimeric fibrinogen assembly with all possible permutations were performed as summarized in Fig. 3. These results showed that the chimeric fibrinogen chains assemble in a chain-specific manner identical to the natural fibrinogen chains, and that the amino-terminal region and the first half of the coiled-coil region exert no influence on the formation of the initial heterodimers. Thus, when chimeric fibrinogen chains consisting of the second half of the coiled-coil and carboxy-terminal domains of the α and γ chains are coexpressed, they assemble in a manner identical to the natural α and γ chains. Similarly, chimeric β and γ chains assemble independently of their amino-terminal and first half of their coiled-coil sequences. However, no assembly of chimeric α and β chains were observed. These latter results are identical to those employing the natural α and β chains. Likewise, various combinations of the amino-terminal sequences of the α and β chains failed to promote assembly. These data indicate that the initial assembly of heterodimeric fibrinogen chains depends on chain-specific interactions of sequences located in the second half of the coiled-coil domain and the carboxyl-terminal domains of each of the three interacting chains.

Assembly of Fibrinogen Chains with Large Internal Deletions—To further assess the importance of the coiled-coil region in the initial assembly process, specific deletions were introduced into the fibrinogen chains. Three sets of conserved intron sites were employed as boundaries between adjacent domains in the construction of these deletion mutants (Fig. 4, A and B). In these studies, three sets of deletion mutants were constructed. These included, 1) deletion of the entire NH2-terminal domain extending to the end of the first half of the coiled-coil domain; the first five residues in the NH2 terminus of each chain, however, was retained as a processing site for signal peptidase; these deletions were αΔ6-102, βΔ6-133, and γΔ6-76; 2) deletion of the first half of the coiled-coil domain in each of the three chains that include the proximal disulfide ring; these included αΔ42-102, βΔ73-133, and γΔ16-76; and 3) deletion of the second half of the coiled-coil domains that include the distal disulfide ring; these were αΔ103-177, βΔ134-209, and γΔ77-151.

Fig. 3. Assembly of fibrinogen chains in cells expressing combinations of normal and chimeric fibrinogen chains. X and Y represent the NH2-terminal and the first half of the coiled-coil regions. +, presence of heterodimers; −, absence of heterodimers.

When the αΔ6-102 and γΔ6-76 chains were coexpressed, or the βΔ6-133 and γΔ6-76 were coexpressed, major complexes formed corresponding to the heterodimers of the mutant αγ or βγ chains (Fig. 5, A and B). Coexpression of three deleted chains led to the formation of the mutant αγ heterodimers and αβγ half-molecules (Fig. 5C). These results confirm the previous data showing that the entire NH2 terminus including the first half of the coiled-coil domain in each of the three chains is not required for the initial assembly of heterodimers or half-molecules. Similar to normal fibrinogen chains, a portion of half-molecules with deleted NH2 termini were also secreted into the medium (data not shown).

Studies on fibrinogen chains with deletions of the first half of the coiled-coil domain and the proximal disulfide ring, but retaining the intact NH2 termini, showed that they also readily participated in the assembly process in a manner similar to the chains with complete NH2-terminal deletions. Thus, coexpression of αΔ42-102 with γΔ16-76, or βΔ73-133 with γΔ16-76 led to the formation of the corresponding mutant heterodimers (Fig. 5, D and E). Coexpression of all three deletion chains gave rise to mutant half-molecules (Fig. 5F). However, the mutant half-molecules were unable to dimerize to generate the six-chain molecule. These results indicate that the NH2-terminal sequence as well as the first half of the coiled-coil domain are required for dimerization of the half-molecules.

Assembly of fibrinogen with deletions in the second half of the coiled-coil domain showed that the major complexes formed were homodimeric molecules with little or no heterodimeric complexes being generated. Thus, coexpression of αΔ103-177 and γΔ77-151, or βΔ134-201 and γΔ77-151 failed to generate heterodimers. Furthermore, no half-molecules were detected when all three deletion mutants, αΔ103-177, βΔ134-209, and γΔ77-151, were coexpressed (data not shown). These results indicated that the second half of the coiled-coil domain is cru-
![Image of a diagram showing the effects of deletions on fibrinogen assembly.](image)

**Fig. 5. Effect of deletions on fibrinogen assembly.** Metabolic labeling, immunoprecipitation, and two-dimensional SDS-PAGE were carried out as described under “Experimental Procedures.” Panels A-C, effect of deletions of the amino termini and the first half of the coiled-coils on fibrinogen assembly; panel A, coexpression of αΔ1-102 and γΔ6-76; panel B, coexpression of βΔ6-133 and γΔ6-76; panel C, coexpression of all three deletion mutants αΔ6-102, βΔ6-133, and γΔ6-76. Panels D-F, effect of deletions of the first half of the coiled-coils and the proximal disulfide ring on fibrinogen assembly; panel D, coexpression of αΔ42-102 and γΔ16-76; panel E, coexpression of βΔ73-133 and γΔ16-76; panel G, coexpression of all three deletion mutants αΔ42-102, βΔ73-133, and γΔ16-76.

The attractive ionic interactions between the γ and α chains, and subsequently the formation of αβγ half-molecules.

**Assembly of Fibrinogen Chains with Point Mutations**—Trimeric coiled-coil sequences display a characteristic periodic heptad repeat of seven amino acid residues (a-b-c-d-e-f-g), with hydrophobic residues present predominantly in positions a and d and polar residues in other positions (Harbury et al., 1993). For dimeric coiled-coils, β-branched amino acids (Ile and Val) are often conserved in positions a, while Leu residues are conserved at positions d of the heptad repeat (Hu et al., 1990). It has also been proposed that for two-stranded coiled-coils, ionic interactions between a charged residue in position g of one heptad in one strand with an opposite charged residue in the e position of a corresponding heptad in the second strand may be important in determining the specificity of two-stranded coiled-coil formation (Conway and Parry, 1990; Beck et al., 1993; John et al., 1994). Two clusters of interhelical g and e ion pairs are present in the second half of the coiled-coil region of the fibrinogen chains (Fig. 6A). The first cluster includes αArg118, βAsp154, γGlu123, and αLys123, while the second cluster involves αAsp146, γLys125, βLys178, αGlu151, γLys151, and αGlu151. To further assess the role of ionic interactions in the second half of the coiled-coil region of the fibrinogen chains in the assembly process, amino acid substitutions that abolish ion pair formation were introduced into the α and γ chains. Hence αR118E and αD146R replacements should change the attractive ionic interactions between the γ and the α chain into a repulsive effect. Similarly, γE92R and γK120E should change the attractive ionic interactions between the γ and the α chain as well as the γ with the β chains to a repulsive effect. At the same time, these replacements should also create a favorable ionic interaction between two α chains, and between two γ chains. If ionic interactions provide the major driving force in the initial assembly process, these replacements would likely favor the formation of homodimers over heterodimers (Fig. 6B). Coexpression of an α chain with single amino acid replacements with normal γ chain, or normal α chain with singly mutated γ chain, led to the formation of αγ heterodimers in amounts indistinguishable from wild type α and γ chains (Fig. 6C, lanes 1–4 and 6). Furthermore, coexpression of the α chain double mutant αR118E/D146R with the γ chain double mutant γE92R/K120E also led to formation of normal amounts of αγ heterodimers (Fig. 6C, lane 5, and D). No increased amount of α chain dimers (αα) or γ chain dimers (γγ) was observed. These results indicated that when all four possible ion pairs were disrupted by amino acid replacements, the formation of αγ heterodimers was still favored. This suggests that the ionic interactions are not essential while hydrophobic interactions between chains are apparently critical in the initial assembly process. The only difference the mutant chains exhibited was that unlike the wild type heterodimers, which were secreted at low levels from the BHK cells, αγ heterodimers with these amino acid replacements were not secreted (data not shown).

**DISCUSSION**

During the synthesis of fibrinogen, the individual α, β, and γ chains are translated, processed, assembled, and eventually secreted into plasma as a six-chain mature fibrinogen molecule. Only very small amounts of partially assembled intermediates, primarily αγ, and αβγ half-molecules, however, are secreted during this process. The synthesis and assembly of fibrinogen has been shown in BHK cells (Farrell et al., 1991), COS-1 cells (Roy et al., 1991; Hartwig and Danishefsky, 1991), and most recently in yeast (Roy et al., 1995), indicating that the assembly process does not require hepatocyte-specific factors. Transient association of the chaperon protein BiP with partially assembled intermediates have been observed in HepG2, BHK, as well as COS-1 cells. Current evidence support a model that the assembly of fibrinogen from its constituent chains follows a stepwise chain addition pathway, although the order of chain addition remains controversial. In one proposal, αβ and βγ complexes were functional intermediates in this process (Roy et al., 1991). However, studies in BHK cells (Huang et al., 1993) and results presented here support the model that only αγ or βγ complexes, but not the αβ complex, are functional intermediates in this process. The preferential formation of these heterodimeric complexes suggests that this specific sequence of chain additions is determined by the inherent amino acid sequences in the fibrinogen chains.

Studies described in this report further identify that the amino acid sequences involved are located in the second half of the coiled-coil domain of the fibrinogen chains. Coiled-coil sequences may adopt a dimeric, trimeric, or an anti-parallel tetrameric conformation, all of which can be accommodated in the “knobs-into-hole” packing model proposed by Crick (1953). According to this model, the side chains (knobs) at positions a and d of one heptad repeat fit into the spaces (holes) between four amino acid residues in a neighboring helix. For two-stranded coiled-coils, β-branched (Ile and Val) are conserved at positions a, while Leu residues are conserved at positions d, whereas for three-stranded coiled-coils, Leu and β-branched amino acids are equally represented in the a and d positions (Conway and Parry, 1991). An examination of the hydrophobic amino acids in the second half of the coiled-coil domain of the three fibrinogen chains shows that Ile and Val residues are...
found predominantly in the α positions and Leu residues are exclusively located at d positions of the heptad repeats. This distribution favors initial two-stranded coiled-coil interactions and is consistent with the model that these regions initiate chain-specific interactions to give rise to an initial intermediate with two-stranded coiled-coils. Furthermore, an examination of the distribution of hydrophobic amino acids in the first half of the heptad repeats shows that Leu and β-branched amino acids (Ile and Val) are evenly distributed in positions a and d of the heptad repeats, representing a distribution that would favor three-stranded coiled-coil formation. This distribution is also consistent with our interpretation that sequences in the first half of the coiled-coil regions participate in a later step in the assembly process, and the formation of the three-stranded coiled-coils can only occur after the initial two-stranded coiled-coils are formed. These data are also consistent with the model that hydrophobic interactions provide the driving force for the initial assembly process as first proposed by Doolittle (Doolittle et al., 1978). On the other hand, mutations in hydrophilic residues have no effect on the assembly process. These hydrophilic interactions may serve to further stabilize the two-stranded coiled-coil after it is formed but are insufficient to bring about the formation of the coiled-coil itself.

The chain-specific ordered assembly process reflects the result of evolutionary selection that parallels the development of other functions in the molecule. The similarity in sequence of the three chains indicates that the three genes evolved from a common ancestor. Before the divergence into multiple chains, the polypeptide from the single primordial gene presumably assembles randomly to form homotrimeric half-molecules and six-chain molecules. After the gene has duplicated and the individual duplicated genes evolve to acquire distinct functions, e.g. polymerization sites, cross-linking sites, and platelet binding sites, a necessity arose that the constituent chains
assemble non-randomly in a chain-specific manner to ensure that the assembled molecule would possess the necessary functional characteristics. Therefore, the selective pressures during evolution have preserved changes in the coiled-coil regions that favor heterodimeric over homodimeric interactions. These changes are not represented in the linear sequence, but in the conservation of hydrophobic amino acids and their distribution, the distribution of \( \beta \)-branched amino acids, the location of disulfide bonds, and the length of the coiled-coil domains.

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