Fibrinogen Assembly and Secretion

ROLE OF INTRACHAIN DISULFIDE LOOPS

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Human fibrinogen is a homodimer composed of three different (Aα, Bβ, γ) polypeptide chains. The chains are linked by 29 inter- and intrachain disulfide bonds. Each half-molecule has 6 intrachain disulfide bonds, which form loops in the carboxyl-terminal region of each of the chains. Aα chain has one disulfide loop (Cys142-Cys472), Bβ has three (Cys201-Cys286, Cys211-Cys240, and Cys394-Cys497), and γ has two loops (Cys153-Cys182 and Cys326-Cys339). The intrachain loops are conserved in fibrinogens of different species. We changed, by site-directed mutagenesis, the cysteines, which form the intrachain loops, to serine or alanine. Fibrinogen chain assembly and secretion was determined in transiently transfected COS cells expressing two normal and a mutant fibrinogen chain. In the Bβ and γ chains, disruption of the disulfide loops closest to the “coiled-coil” region (CysB211-Cys240, CysB201-Cys286, and Cysγ153-Cys182) abolished chain assembly and secretion, indicating that the disulfide loops closest to the coiled-coil region are essential for assembly. By contrast, preventing formation of the disulfide loops, which are toward the carboxyl termini of each of the chains, had different effects. Disruption of the single Aα disulfide loop had no effect, as did disruption of BβCysB394-Cys497. However, disruption of Cysγ326-Cys339, which is similar in size and location to CysB284-Cys307, allowed chain assembly to occur, but the assembled chains were not secreted.

Human fibrinogen is a dimer with each half-molecule composed of three different polypeptide chains: Aα, Bβ, and γ (for reviews, see Refs. 1–3). Structural studies indicate that fibrinogen is elongated and trinodal. The central node (E domain) consists of the amino termini of the six polypeptide chains, and the terminal nodes (D domain) are formed by globular carboxyl-terminal domains of Bβ and γ. The carboxyl-terminal ends of the large Aα chain are thought to fold back and contribute to the structure of the central node. The central E domain is connected to the terminal D domain by a 3-chain α-helical coiled-coil structure (4–8). Fibrinogen is stabilized by 29 inter- and intrachain disulfide bonds, which include 17 interchain disulfide bonds and 12 intrachain disulfide connections (9–13). Each half-molecule has six intrachain disulfide bonds, which form loops in the carboxyl-terminal region of each of the chains. Aα chain has one disulfide loop (Cys142-Cys472), Bβ has three (Cys201-Cys286, Cys211-Cys240, and Cys394-Cys497), and γ has two intrachain loops (Cys153-Cys182 and Cys326-Cys339). The locations of the intrachain disulfide loops in the globular domains of the Bβ and γ chains have been conserved during evolution and are similar in diverse species such as human and lamprey (14). A diagram depicting the locations of the inter- and intrachain disulfide bonds of fibrinogen is shown in Fig. 1.

Previous studies demonstrated the importance of amino-terminal interchain disulfide bonds on assembly of the two half-molecules to form 6-chain fibrinogen (15–17) and indicated that, in addition to the symmetrical disulfide bonds that link adjacent AαCys284 and Cys394 and Cysγ153 of one half-molecule to the other half-molecule, another disulfide interaction, CysB495 to Cysγ336, also connects the two half-molecules of fibrinogen (15, 17). Disruption of all of the disulfide interactions that connect the two half-molecules leads to the assembly and secretion of half-molecules and not of fully formed fibrinogen. However, the interchain disulfide rings at the amino-terminal side of the coiled-coil region (Fig. 1, arrow marked N) are also essential for dimerization of the two half-molecules. Maintaining all of the cysteine residues involved in disulfide linkage of the half-molecules (γ8 and 9, Aα28 and 36, and Bβ65), but disrupting the disulfide rings that flank the amino-terminal side of the coiled-coil region, inhibits dimerization, and, again half-molecules but not fibrinogen are formed and secreted. On the carboxyl-terminal side of the coiled-coil region the interchain disulfide rings (Fig. 1, arrow marked C) are not needed for chain assembly, but if they are not present, fibrinogen is assembled but is not secreted, probably due to improper folding (15).

The present studies focus on the role of intrachain loops in fibrinogen assembly and secretion (Fig. 1, shaded areas). We changed, by site-directed mutagenesis, the cysteines that form intrachain loops to serine or alanine and investigated in transiently transfected COS cells the assembly and secretion of fibrinogen containing the mutant chain.

EXPERIMENTAL PROCEDURES

Materials—Full-length Aα, Bβ, and γ cDNAs, cloned into the PstI site of pBR222, were kind gifts from Dr. Dominic Chung (University of Washington, Seattle). Expression vector pED4-Neo (18) was obtained from the Genetics Institute (Cambridge, MA). Other reagents used have been described (15, 19–23).

Plasmid Construction and Mutagenesis—Full-length Aα, Bβ, and γ cDNAs were subcloned into M13mp18 or M13mp19 (15, 16, 24). To construct pED4-NeoAα, pED4-NeoBβ, and pED4-Neoγ, EcoRI sites were introduced into the 5′-end of Aα cDNA of M13mp19Aα, the 5′-side of PstI of M13mp19Bβ, and the 5′-3′-ends of γ cDNA of M13mp19γ by site-directed mutagenesis (25). The following oligonucleotides were used: CTTTTCTAGAATTCGAGCTGCT, GACCTGACAGGATCCATCAGCTATC, and CCCTCCGAGGATCCATGTC. To eliminate an internal EcoRI site in Aα cDNA, an oligonucleotide (GGAAGGGAACTCAGCTATC) was used to change the nucleotide sequences GAA to GAG without changing glutamic acid at position 550. Each of the full-length cDNAs was excised by digestion with EcoRI and inserted into the EcoRI site of expression vector pED4-Neo. The correct orientation was selected by restriction

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cloned into g

tained in Iscove's medium containing 10% fetal calf serum and 1%

pED4-Neo. All mutations and correct orientations of mutant cDNAs in
codon GCT. The mutant cDNAs were subcloned into
(TGTTCATC) was made that changed cysteine codon TGT to alanine

inserted into the
Eco RI site of pED4-Neo. The correct orientation of
mutant cDNAs was confirmed by DNA sequencing.

Mutants of Aα Chain—Mutant AαC472S (which encodes a serine
instead of cysteine at position Aα247) was made by changing cysteine

codon TGT in single strand DNA of M13mp18Aα to serine codon AGT.
A double mutant AαC442S,C472S, which encodes cysteine to serine
substitutions at amino acid positions 442 and 472 of Aα chain, was also
similarly prepared. Two synthetic oligonucleotides, GCCACGGCTT-
GAAAGACCCG for AαC442S and GCTCTGGAGCTTGTGAGACCGA for
AαC472S, were used. Site-directed mutagenesis was performed accord-
ing to the method of Kunkel (25). The mutations were confirmed by
DNA sequencing (26). All mutant cDNAs were digested with EcoRI and
inserted into the EcoRI site of pED4-Neo. The correct orientation of
mutant cDNAs was confirmed by DNA sequencing.

Mutants of Bβ Chain—Single or double mutants of the Bβ chain were
made as described previously (16, 24). Four mutants, BβC201S,C286S,
BβC240S, BβC211S,C240S, and BβC394S,C407S, were constructed using
oligonucleotides GGAATATTGCTACTGACAGTG (for BβC201S),
CTGGTTAGCCTGCTGTTCTTC (for BβC286S), ATTCCTCTCCT-
CTCTGCG (for BβC211S), TTATGGCTCTGCTATCTTA (for BβC240S),
GCGCTCTGCTTGTGAGACCT (for BβC394S), and GCGCTCCTGCTTAT,-
TAC (for BβC407S) by changing cysteine codon (TGC or TGT) to serine

codon (AGT or AGC). All mutations were confirmed by DNA
sequencing. Full-length cDNAs of all mutants were released from M13mpBβ
digested by EcorI and subcloned into EcoRI site of pED4-Neo.

Mutants of γ Chains—To construct γ chain mutants, single strand
DNA of M13mp18γγ) was synthesized as a template. Four mutants that
encode cysteine to serine substitutions were constructed: 1) γγC153S,C182S,
2) γγC286S, 3) γγC339S, and 4) γγC394S,C407S. Oligonucleotides
AGTCTAGTGCTAGCTACCTCTCATC (for γγC153S), TCGAGTT-
CCAGACGC (for γγC286S), CGCTGGCTGCTGCTATCTTA (for
γγC339S), and GCCAGCTGCTGCTGCTACCT (for γγC394S,C407S) were
used. To construct a mutant γγC339A, which would encode alanine
instead of cysteine at γγ339, an oligonucleotide (GCCAGCTGAGCCCT-
GCTACCT) was made that changed cysteine codon TGT to alanine

codon GCT. The mutant cDNAs were subcloned into EcoRI site of
pED4-Neo. All mutations and correct orientations of mutant cDNAs in
expression vector pED4-Neo were confirmed by DNA sequencing.

Expression of Mutant Chains—The expression of mutant fibrinogen chains was compared with that of normal chains by
transfection in COS cells. Nascent fibrinogen chains were iso-
lated from radiolabeled cell lysates using polyclonal antibody to
human fibrinogen, and the radioactive proteins were separated by
SDS-PAGE (Fig. 2). All of the mutant chains were expressed at about the same level as the normal chains (Fig. 2A). Four
mutant chains, BβC201S,C286S, BβC394S,C407S, γγC339S,
and γγC326S,C339S (marked by asterisks), migrated slower on
SDS-PAGE than normal Bβ and γ chains, and two of them,
γγC339S and γγC326S,C339S, migrated as doublets (Fig. 2A,
lanes 5, 8, 12, and 14). Changing cysteine to serine at amino
acid positions 286 and 407 of Bβ chain or at amino acid position
339 of γ chain creates a possible extra N-linked glycosylation site
(Aasn-X-Ser). To determine if the apparent increase in molecu-
lar weight resulted from an extra N-linked oligosaccharide chain,
another mutant (γγC339A), which encodes for alanine
instead of serine, was constructed. Alkaline at amino acid position
339 of γ chain does not create an extra N-linked glycosylation
site. In contrast to γγC339S, γγC339A (Fig. 2A, lane 13)
migrated on SDS-PAGE as a single band, with similar mobility to
normal γ chain.

Immunoprecipitation of nascent fibrinogen was carried out as de-
scribed previously (15, 16, 19, 24). The labeled cell lysates and
the incubation medium were immunoprecipitated using a rabbit polyclonal
antibody to human fibrinogen (Dako Corp.) and protein A-Sepharose.

Immunoprecipitates were analyzed under reduced and non-reduced
conditions and detected by autoradiography.

Characterization of Secreted Fibrinogens—Wild-type and mutant fi-
brinogens, which were secreted into the incubation media, were char-
acterized, as described previously (23), by two-dimensional SDS-
PAGE.1 The first dimension was non-reduced, and the second
dimension was with reduced gels (data not shown).

RESULTS

Expression of Mutant Chains—The expression of mutant fibrinogen chains was compared with that of normal chains by
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migrated on SDS-PAGE as a single band, with similar mobility to
normal γ chain.

Previous studies showed that small amounts of single Aα and
γ chains could be secreted by transfected COS cells (23).
Therefore, we compared the secretion of single Aα and
γ chains to the normal Aα and γ chains. A small amount of
normal Aα, AαC472S, and AαC442S,C472S were detected in the
culture media on prolonged exposure of the autoradiograms
(Fig. 2B, lanes 1–3). Larger amounts of normal γ chain was
secreted (Fig. 2B, lane 9), but the mutant γ chains were not
secreted, indicating that intact intrachain loops may be necessary
for secretion of the γ chain (Fig. 2B, lanes 10–14). Neither the normal nor the mutant Bβ chains were secreted (Fig. 2B,

1The abbreviation used is: PAGE, polyacrylamide gel

electrophoresis.
Substitutions at the indicated positions. In mutant chains, cysteine to serine (S) or cysteine to alanine (A) that migrate aberrantly on SDS-PAGE are marked by asterisks.

The autoradiogram in panel A indicates the wild-type fibrinogen chains. The proteins were separated by SDS-PAGE and detected by immuneprecipitation with a polyclonal antibody to human fibrinogen. The proteins were metabolically radiolabeled for 2 h with L-[^35]S]methionine, and radioactive fibrinogen present in the cell lysate (panel A) and secreted into the medium (panels B and C) was isolated by immunoprecipitation and SDS-PAGE and detected by autoradiography. Panels A and B are non-reduced gels, and panel C contains reduced fibrinogen. aβγ (lane 1) is wild-type fibrinogen, and a472S and a442S,472S indicate that cysteine to serine substitutions occurred in those amino acid positions. The locations of fibrinogen (Fb), aα, and aβγ complexes and of free chains (a, β, and γ) are indicated. aC472S and aC442S,472S migrated slower on SDS-PAGE and are marked (α°).

**Fig. 2.** Expression of mutant fibrinogen chains. Wild-type and mutant fibrinogen chains were expressed in COS cells, and the metabolically radiolabeled fibrinogen chains were isolated from cell lysates by immunoprecipitation with a polyclonal antibody to human fibrinogen. The proteins were separated by SDS-PAGE and detected by autoradiography. α, β, and γ indicate the wild-type fibrinogen chains. The mutant chains contain cysteine to serine (S) or cysteine to alanine (A) substitutions at the indicated positions. In panel A, the mutant chains that migrated abnormally on SDS-PAGE are marked by asterisks. Panel B contains protein secreted into the cell medium after 2 h of incubation. The autoradiogram in panel A was exposed for 4 h and that in panel B for 6 days.

An Intact Intrachain Loop of Aa Chain Is Not Necessary for Fibrinogen Assembly and Secretion—Fibrinogen Aa chain has only one intrachain loop in the carboxyl-terminal domain spanning Cys442 to Cys472. To determine if this Aa intrachain loop is required for fibrinogen assembly and secretion, a mutant containing a single Cys to Ser substitution (AaC472S) and another with two substitutions (AaC442S,472S) were made. COS cells were cotransfected with the mutant Aa chain cDNA and with normal Bβ and γ chains cDNAs. Disruption of the Aa intrachain loop by substituting cysteine with serine at amino acid position 472 or in both positions (442 and 472) did not affect fibrinogen assembly and secretion. In both cases, the Aa mutant chains were assembled with the wild-type Bβ and γ chains forming, and accumulating, fibrinogen and Aαγ complex intracellularly (Fig. 3A, lanes 2 and 3). Small amounts of Bβγ complex and of free Aa, Bβ, and γ chains were also detected intracellularly. There was less free AaC472S (Fig. 3A, lane 2) than free AaC442S,472S or normal Aa (Fig. 2A, lanes 1 and 3). Mutants AaC472S or AaC442S,472S (marked by circles) had slower migration on SDS-PAGE, under non-reduced conditions, than normal Aa (Fig. 3A, lanes 2 and 3). This may be the result of a change in conformation resulting from loss of the intrachain disulfide loops since mutants AaC472S or AaC442S,472S migrated similarly to normal Aa chain under reduced conditions (Fig. 2, lanes 2 and 3).

The mutant fibrinogens, containing either AaC472S or AaC442S,472S were secreted (Fig. 3, B and C, lanes 2 and 3) in near equal amounts to wild-type fibrinogen (Figs. 3, B and C, lane 1).

**Fig. 3.** Aa intrachain disulfide loop. Transfected COS cells were metabolically labeled for 2 h with L-[^35]S]methionine, and radioactive fibrinogen present in the cell lysate (panel A) and secreted into the medium (panels B and C) was isolated by immunoprecipitation and SDS-PAGE and detected by autoradiography. Panels A and B are non-reduced gels, and panel C contains reduced fibrinogen. aβγ (lane 1) is wild-type fibrinogen, and a472S and a442S,472S indicate that cysteine to serine substitutions occurred in those amino acid positions. The locations of fibrinogen (Fb), aα, and aβγ complexes and of free chains (a, β, and γ) are indicated. aC472S and aC442S,472S migrated slower on SDS-PAGE and are marked (α°).
Fibrinogen Assembly

**DISCUSSION**

The fibrinogen intrachain loop domains are conserved in different species, suggesting that they play important roles in maintaining structures which allow biological functions. The
The strictest conservation of amino acid sequence and location of the intrachain loops are in the Bβ and γ chains. The intrachain loops close to the coiled-coil region show remarkable similarities in size and location in human, chicken, Xenopus, and lamprey fibrinogen (14). Interestingly, similar intrachain loops are found in fibrinogen-related proteins such as tenascin (27), the Sca protein in Drosophila (28), and in some other proteins (29). In the Aα chain, a single intrachain loop (Cys442–Cys472 in human fibrinogen) is similar in size and in amino acid sequence in nine other species (30). The Aα chain intrachain loop is missing, however, in lamprey fibrinogen. Lamprey also expresses a minor form of the Aα chain whose carboxyl-terminal half is homologous to the carboxyl-terminal globular domain of the Bβ and γ chains and contains two intrachain disulfide loops (14). Humans also express, in small quantity, a fibrinogen with an extended Aα chain (α5γ), and α5γ also contains two intrachain disulfide loops that are similar to those in Bβ and γ chains (31, 32). In this study, we demonstrate that the conserved intrachain loops, particularly those in the Bβ and γ chains in the predominant form of human fibrinogen, play important roles in fibrinogen assembly and secretion.

A prominent feature of fibrinogen is an α-helical coiled-coil domain spanning about 111 hydrophobic amino acids in each of the chains and flanked on either end by a pair of cysteine residues (Cyx-X-X-Cyx), which form interchain disulfide linkages and are commonly termed “disulfide rings” (3, 33). The coiled-coil region links the central E domain to the terminal D domain, providing fibrinogen with its well-known elongated, trinodal structure. Our present studies show that not only the interchain disulfide bonds but also some of the intrachain disulfide interactions are necessary for assembly and secretion (see Fig. 6 for summary). Preventing formation of the intrachain disulfide loops closest to the coiled-coil region (Cysββ201, Cysγ286, Cysββ221–Cysγ240, and Cysγ153–Cysγ182) does not allow fibrinogen to be assembled, and, in these conditions, neither the 3-chain (αα-Bβγ) complex nor the 6-chain fibrinogen is formed. Preventing formation of these intrachain disulfide bonds may change the conformation of the chains so that complexes larger than two chains cannot be assembled. These Bβ and γ chain mutants, however, form Bβγ and ααγ complexes, although the formation of these 2-chain complexes appears to be also inhibited. Fibrinogen assembly is a stepwise process, proceeding from single chains to 2-chain complexes...
(predominantly Aαγ and Bβγ) to 3-chain half-molecules (AαBβγγ), which dimerize to form fibrinogen (AαBβγγ). Disruption of the Bβ and γ intrachain loops closest to the coiled-coil region appears to mostly affect the addition of a third chain to Aαγ and to Bβγ complexes, thus inhibiting the formation of AαBβγγ half-molecules. The mechanisms by which the 3-chain coiled-coil region is assembled and stabilized by the disulfide rings is not well understood, but these studies suggest that, in addition to the amino acid sequence responsible for α-helix formation and the flanking interchain disulfide rings, the Bβ and γ intrachain loops closest to the coiled-coil region are also needed to allow proper alignment and formation of the 3-chain coiled-coil structure.

In addition to the intrachain loops close to the coiled-coil region, each of the chains has distal intrachain loops toward the carboxyl terminus (CysAα194-CysAα407, CysBβ184-CysBβ407, and Cysγγ194-Cysγγ472). CysBβ194-CysBβ407 and Cysγγ194-Cysγγ472 are similar in size and in location and are different from CysAα442-CysAα472, which spans a larger amino sequence (see Figs. 1 and 6). Disruption of these carboxyl-terminal disulfide loops have different effects on fibrinogen assembly and secretion. CysAα442-CysAα472 may be very important in fibrinogen function since this domain is conserved in different species, but disruption of this disulfide loop has little or no effect on chain assembly and secretion. On the other hand, disruption of CysBβ194-CysBβ407 also does not block fibrinogen assembly and secretion but does influence the assembly process and leads to intracellular accumulation of Aαγ complex, some of which is secreted. By contrast, disruption of Cysγγ194-Cysγγ472 allows fibrinogen assembly but completely blocks secretion. This shows that intact Cysγγ194-Cysγγ472 is not necessary for fibrinogen chain assembly, but if this intrachain loop is not formed, the structure of fibrinogen is sufficiently altered so as to prevent it from being secreted. It should be noted that small amounts of free γ chains are secreted by transfected COS cells (Fig. 2B) and that preventing formation of the intrachain disulfide loops also abolishes secretion of the free γ chain.

A previous study showed that a truncated Bβ chain, lacking the carboxyl-terminal amino acids 208–461, could assemble with wild-type Aα and γ chains, and the mutant fibrinogen was secreted (24). Since the deleted segment contains all three Bβ intrachain loops, this suggested that this domain is not necessary for assembly and secretion. Yet, our present studies demonstrate that the intrachain loops CysBβ201-CysBβ286 and CysBβ211-CysBβ240, which are present in this region, are needed for fibrinogen assembly. Taken together, this indicates that a truncated Bβ chain containing amino acids 1–207, which includes the coiled-coil region and the flanking disulfide rings but lacks the carboxyl-terminal domain, is sufficient for assembly; but if the entire Bβ chain is expressed, then the intrachain loops are necessary to maintain the structure of the carboxyl-terminal domain so that it does not interfere with formation of the coiled-coil region.

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