Symmetrical Disulfide Bonds Are Not Necessary for Assembly and Secretion of Human Fibrinogen*

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Human fibrinogen is a dimer, and each half-molecule is composed of three different polypeptides (Aα, Bβ, and γ). The two half-molecules are joined together at the amino-terminal ends by three symmetrical disulfide bonds between adjacent Aα chains, at position 28, and between adjacent γ chains at γ8 and 9. To determine the role of these disulfide bonds in fibrinogen assembly and secretion, site-directed mutagenesis was used to change cysteines of Aα28 and γ8 and 9 to serine, the mutant chains were coexpressed with normal fibrinogen chains in COS cells, and assembly and secretion of fibrinogen were measured. Elimination of the symmetrical disulfide bonds did not affect assembly of the chains and dimeric fibrinogen was secreted. Analysis by plasmin digestion indicated that the secreted mutant fibrinogens have a similar structure to normal fibrinogen. Our results indicate that other domains of fibrinogen participate in dimer formation and that the three symmetrical disulfide bonds are not crucial for assembly and secretion of fibrinogen.

Our previous studies showed that in Hep G2 cells nascent fibrinogen chains are assembled in the rough endoplasmic reticulum into dimeric fibrinogen (7), and pulse-chase studies suggested that chain assembly occurred in a step-wise fashion, beginning with the attachment of preformed Aα and γ chains to nascent Bβ chains forming two-chain complexes, followed by the addition of a third chain to form half-molecules, which then join together to form dimeric fibrinogen (8–10). However, the detailed mechanism of fibrinogen assembly, at the molecular level, has not been elucidated. To study these events we have used transfected COS cells which coexpress Aα, Bβ, and γ chains and are capable of assembling and secreting fibrinogen (11). Previously we showed that Bβ domain between amino acids 73 and 93 is necessary for assembly of the three fibrinogen chains and that the Bβ 1–72 is involved in the formation of a six-chain dimeric molecule (12). In this study, we have modified the three symmetrical disulfide bonds (Aα28, γ8, and 9), which join the two half-molecules of fibrinogen and studied the assembly and secretion of fibrinogen in transiently transfected COS cells.

EXPERIMENTAL PROCEDURES

Materials

Plasmin was purchased from Kabi Vitrum, endoglycosidase H from Genzyme, l-[35S]methionine from Du Pont, and rabbit antibody to human fibrinogen from DAKO Corp. Full-length Aα, Bβ, and γ cDNA, cloned into the PstI site of pBR322, were kind gifts from Dr. Dominic Chung, University of Washington, Seattle. The vectors containing full-length Aα, Bβ, and γ cDNA, (pBC12BI-Aa, pBC12BI-
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Expression of Mutant Fibrinogen Chains—To examine the expression of mutant fibrinogen chains, COS cells were transfected with pBC12BI-Aα(28s), pBC12BI-γ(8s,9s), pBC12BI-Aα, or pBC12BI-γ and metabolically labeled with L-[^35]S methionine. The radioactive fibrinogen chains were immunoprecipitated with polyclonal antibody to human fibrinogen and analyzed by SDS-PAGE. Mutant Aα(28s) was expressed at a similar level to normal Aα chains and had identical mobility on SDS-PAGE. Both the normal and the mutant Aα chains were extensively degraded during isolation (Fig. 2, lanes 4 and 5). Mutant γ(8s,9s) yielded a double band. The lower band had the same electrophoretic mobility as normal γ chain (Fig. 2, lanes 2 and 3). The estimated differences in molecular mass between normal γ chain and the top band of mutant γ(8s,9s) chain is approximately 3000 Da.

Mutant γ Chain (γ8s,9s) Contains an Extra N-Linked Oligosaccharide—Changing cysteine residues 8 and 9 to serine creates a possible extra N-linked glycosylation site (Asn-Ser) on the γ chain. To determine whether the larger γ chain contained an extra N-linked oligosaccharide chain, cell lysates from transfected COS cells expressing normal and mutant γ(8s,9s) chains were digested with endoglycosidase-H and analyzed on SDS-PAGE. Endoglycosidase-H cleaves N-linked mannose-rich oligosaccharides but does not remove N-linked oligosaccharides which have been trimmed of mannose and further processed in the Golgi complex (18, 19).

Normal γ chain, not treated with endoglycosidase-H, when expressed alone migrated on SDS-PAGE as a 49-kDa protein (Fig. 3, lane 1). On treatment with endoglycosidase-H the N-linked mannose-rich oligosaccharides were removed and the γ chain migrated faster (Fig. 3, lane 2). The mutant γ chain (γ8s,9s) migrated as a larger protein, mostly as a 52-kDa protein, when not treated with endoglycosidase-H (Fig. 3, lane 3). On treatment with endoglycosidase-H γ8s,9s migrated faster; most of it had the same electrophoretic mobility as

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endoglycosidase-H-treated normal γ chain and some of it migrated similarly to untreated normal γ (Fig. 3, lane 4). This indicates that γ(8s,9s) contained mannose-rich N-linked oligosaccharides and that on removal of these sugar residues, the mutant γ chain acquires the same molecular weight as normal γ chain.

Further evidence that the larger molecular mass of γ(8s,9s) is due to extra N-linked oligosaccharides was obtained by coexpressing γ(8s,9s) together with normal Aa and γ chains, thus allowing chain assembly and secretion to occur. On treatment of intracellular normal Aa, Bβ, and γ chains with endoglycosidase-H, the Bβ and γ chains were cleaved and migrated faster. The migration of Aa, which is not a glycoprotein, was not affected (Fig. 3, lanes 5 and 6). Coexpression of normal Aa and Bβ together with γ(8s,9s) showed the expected pattern of γ(8s,9s) migrating slower than normal γ (Fig. 3, lane 7). Endoglycosidase-H treatment of this sample again did not affect the migration of Aa, but cleaved both Bβ and γ(8s,9s), with γ(8s,9s) acquiring the same mobility as endoglycosidase-H-treated normal γ chain (Fig. 3, lane 8).

Endoglycosidase-H treatment of secreted fibrinogen chains (Fig. 3, lanes 9–12) did not cleave the sugar residues of Bβ, γ, and γ(8s,9s) chains, which is to be expected if the N-linked oligosaccharides are fully processed. This demonstrates that γ(8s,9s) can be assembled with normal Aa and Bβ chains and is secreted together with these chains and that the N-linked sugars, including the extra oligosaccharide, are processed.

Disruption of the Three Symmetrical Disulfide Bonds (Aα 28 and γ 8 and 9) Does Not Affect Dimeric Fibrinogen Assembly and Secretion—To examine the role of the three symmetrical disulfide bonds (Aα 28 and γ 8 and 9) in chain assembly and secretion, mutant Aαγ28s or γ(8s,9s) chains were coexpressed with normal fibrinogen chains in COS cells. The fibrinogen chain complexes present in the cell lysates and also those secreted into the incubation medium were immunoprecipitated and analyzed under reduced and non-reduced conditions by SDS-PAGE (Fig. 4). COS cells coexpressing normal Aα, Bβ, and γ chains assemble and secrete fibrinogen (11). Intracellularly the principal fibrinogen products are dimeric fibrinogen, Aα-γ complex and free Aα, Bβ, and γ chains. The amount of free γ chains was less than that of Aa and Bβ (Fig. 4, lane 1). COS cells coexpressing mutant Aαγ28s and normal Bβ and γ chains displayed a similar pattern. The principal fibrinogen-related products were a protein which migrated similarly to dimeric fibrinogen (Mr 340,000), Aα-γ complex and free Aαγ28s, Bβ, and γ chains (Fig. 4, lane 2). Similar intracellular patterns were also obtained when COS cells coexpressed normal Aα, Bβ, and mutant γ(8s,9s) chains (Fig. 4, lane 3) or mutant Aαγ28s, normal Bβ, and mutant γ(8s,9s) chains (Fig. 4, lane 4). The only difference is that when mutant γ(8s,9s) chains were expressed, the free mutant chain γ(8s,9s), as described earlier, had a different electrophoretic mobility due to extra glycosylation than normal γ chain.

The secreted radioactive proteins, present in immunoprecipitates obtained from the incubation media, were analyzed by SDS-PAGE in reduced (Fig. 4, lanes 5–8) and non-reduced (Fig. 4, lanes 9–12) conditions. The medium from COS cells expressing normal Aα, Bβ, and γ chains contained, in reduced conditions, free Aα, Bβ, and γ chains and higher molecular weight proteins (Fig. 4, lane 5). The higher molecular mass radioactive proteins migrated at the top of the gel, above and below the 200-kDa marker, and as a 97-kDa protein. A similar radioactive pattern was obtained from cells expressing mutant Aαγ28s and normal Bβ and γ chains (Fig. 4, lane 6). In cells expressing normal Aα and Bβ and γ(8s,9s) (Fig. 4, lane 7) or Aαγ28s, normal Bβ, and γ(8s,9s) (Fig. 4, lane 8), the pattern was different because γ(8s,9s) migrated as a doublet, with the larger molec-
ular weight product containing the extra N-linked oligosaccharide.

In non-reduced conditions, only large molecular weight complexes were immunoprecipitated from the incubation medium (Fig. 4, lanes 9-12). Three areas were noted; the top region, marked Fb in Fig. 3, lanes 9-12, coincided with the location of authentic fibrinogen and two lower bands, marked by arrows, were located below fibrinogen but above the 200-kDa marker. These areas were excised, reduced, and re-electrophoresed. The top bands, which migrated in the same location as authentic 340,000-dalton fibrinogen, yielded the expected three component chains of fibrinogen. They contained either normal Aα, Bβ, and γ chains or normal Bβ with the expected mutant Aα or γ chains. The other two high molecular weight areas did not produce lower molecular weight components on reduction (data not shown). This indicates that mutant Aα(28s) and γ(8s,9s) can assemble with normal fibrinogen chains to form a high molecular weight complex similar to fibrinogen and that this complex is secreted. There was no indication that a half-molecule of fibrinogen, composed of only three chains, was secreted. The incubation medium however also contained contaminating high molecular weight proteins that coprecipitate with fibrinogen.

**Plasmin Digestion of Normal and Mutant Fibrinogens—** Fibrinogen is progressively digested by plasmin. The initial product is fragment X and later fragments Y, D, and E are formed (20-22). This pattern of fibrinogen digestion by plasmin reflects structural features of fibrinogen and can only be obtained with a six-chain dimeric molecule. To compare the structure of mutant and normal fibrinogens, the secreted fibrinogens from transfected COS cells expressing normal and mutant chains were digested by plasmin in the presence of CaCl₂ or EDTA. As a control, purified human plasma fibrinogen was also treated with plasmin. The digestion products were isolated by immunoprecipitation and analyzed on SDS-PAGE (Fig. 5).

Normal and mutant fibrinogens, secreted by transfected COS cells, migrated as dimeric fibrinogen (M, 340,000) under non-reduced conditions (Fig. 5B, lanes 1, 4, and 7). On treatment with plasmin in the presence of calcium, fragments D1 and D3 and a protein with an approximate molecular mass of 50 kDa were the principal products (Fig. 5B, lanes 2, 5, and 8). In the presence of EDTA (Fig. 5B, lanes 3, 6, and 9), the D1 fragment disappeared, and the principal products were D3 and the 50-kDa band.

To determine the component chain fragments in D1, D3, and the 50-kDa product, the bands were excised from the gels, reduced, and re-electrophoresed on SDS-PAGE (data not shown). D1 yielded 43, 38, and 12 kDa bands, whereas D3 was composed of 43, 25, and 12 kDa products. These results are consistent with the observations of Nieuwenhuizen and Haverkate (23) and identify these bands as D1 and D3. The 50-kDa band had four components of 43, 23, 18, and 12 kDa, suggesting that the 50 kDa band may be a mixture of fragment E and further digestion products of fragment D.

As a control, purified plasma fibrinogen was also treated with plasmin. Untreated fibrinogen ran at the top of the gel as a 340,000-dalton protein and, since it was immunoprecipitated, the SDS-PAGE gels also contained large amounts of IgG which migrated between the 200- and 97-kDa molecular mass markers (Fig. 5A, lane 1). Treatment with plasmin, in the presence of CaCl₂, digested fibrinogen and lower molecular weight products. Principally D1 was formed (Fig. 5A, lane 2). On treatment with plasmin in the presence of EDTA, D1 disappeared and D3 and a 50 kDa band were produced (Fig. 5A, lane 3). This pattern is similar, but not identical, to that obtained with the recombinant fibrinogens (Fig. 5B).

**Fig. 5. Plasmin digestion of normal and mutant fibrinogen.** Transfected COS cells were incubated with L-[³⁵S]methionine for 16 h at 37 °C. The labeled incubation medium was digested by plasmin in the presence of 10 mM CaCl₂ or EDTA, followed by immunoprecipitation with polyclonal antibody to human fibrinogen. Immunoprecipitates were separated under non-reduced conditions in 7.5% SDS-PAGE. Panel A shows gels, stained with Coomassie Brilliant Blue, of purified plasma fibrinogen digested with plasmin. Panel B shows an autoradiogram of plasmin digestion products of fibrinogens secreted by transfected COS cells. In panel B, lanes 1-3 contain recombinant fibrinogen with normal chains; lanes 4-6, fibrinogen with normal Bβ and γ and mutant Aα(28s); and lanes 7-9, fibrinogen with Aα(28s), normal Bβ, and γ(8s,9s). The incubation conditions, indicating the presence or absence of plasmin, CaCl₂, or EDTA are shown at the top of each lane.

**DISCUSSION**

Fibrinogen is a dimeric molecule with two identical halves. Blomback et al. (6) have shown that the half-molecules are linked by three symmetrical disulfide bridges, two between adjacent γ chains (γ8 and 9) and one between adjacent Aα chains (Aα28). How the NH₂ termini of the three pairs of non-identical chains interact to form the central domain of fibrinogen is not well understood although the fact that this
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domain contains 11 disulfide bonds, including the three symmetrical bonds (5, 6), suggests that disulfide interactions may play a role in assembling and maintaining this portion of the fibrinogen molecule. In this study we show that the three symmetrical disulfide bonds which link the two half-molecules are not essential for fibrinogen chain assembly and secretion.

Procyk et al. (24) have shown that partial reduction of fibrinogen, by dithiothreitol, in calcium-free buffer cleaves two intra-chain disulfide bonds (Aα 442-Aα472 and γ326-γ339) and also the three symmetrical disulfide bonds between adjacent γ8 and 9 and Aα28. Disruption of the symmetrical disulfide bonds did not yield fibrinogen half-molecules even when the partially reduced fibrinogen was treated with 6 M guanidine hydrochloride or 5% acetic acid or when the partially reduced fibrinogen was analyzed on SDS-PAGE. This suggests that other, non-covalent, interactions are involved in linking the half-molecules of fibrinogen and contribute to the formation of the central domain. Complete reduction, of course, shows disassembly of fibrinogen into component chains when analyzed by SDS-PAGE.

There are other examples of multi-chain proteins linked by disulfide bonds that are not essential for chain interactions. For example, Mejers et al. (25) have demonstrated that changing cysteine 321 of factor XI to serine did not abolish dimer function. This indicates that the interchain disulfide at cysteine 321 is not essential for Factor XI dimeric protein to be formed, and the study suggested that another domain (apple 4) mediates dimerization of the two subunits.

Our previous studies showed that deletion of the first 72 NH2-terminal amino acids of Bβ chain did not prevent assembly of a three-chain complex, but it did markedly inhibit the formation of the six-chain dimeric molecule. The main secretory product was a three-chain half-molecule consisting of normal Aα and γ chains with truncated Bβ Δ1–72 (12). Taken together with the present study this indicates that the NH2-terminal domains of all three chains may be necessary, and sufficient, for dimer formation and that the three symmetrical disulfide bonds may be linked after the six chains have assembled. The symmetrical disulfide bonds may function in a secondary role to stabilize the dimer in a distinct configuration.

CO2 cells which express mutant fibrinogens with cysteine to serine substitutions at Aα28, γ8, and γ9 secrete a high molecular weight product into the medium, indicating that the mutant fibrinogens are probably sufficiently well folded to be recognized as a normal protein by the secretory mechanism. Treatment of the mutant fibrinogens with plasmin yielded fibrinogen degradation products which can only be obtained if the mutant fibrinogens are arranged in similar domains as normal plasma fibrinogen. This information, together with the fact that the mutant fibrinogens are secreted under non-reduced conditions as a 340,000-dalton complex, indicates that a dimeric six-chain molecule is assembled and secreted. We do not know, however, whether replacement of cysteine by serine at Aα28 and γ8 and 9 may cause other disulfide bonds in fibrinogen to rearrange or whether the mutant fibrinogens are biologically active.

It is of interest to note that when cysteine at position 9 in the γ chain was replaced by serine a new N-linked glycosylation site was produced and that the nascent γ chain acquired an extra oligosaccharide. The addition of extra sugar residues did not impair chain assembly or secretion. Our earlier studies demonstrated that inhibition of N-linked glycosylation also has no effect on chain assembly and secretion (8). It is of interest to note that several congenital dysfibrinogens have been found which produce additional N-glycosylation consensus sequences. These dysfibrinogens have extra N-linked oligosaccharides and lead to abnormal fibrinogen functions (26–28).

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