Biosynthesis of Human Fibrinogen
SUBUNIT INTERACTIONS AND POTENTIAL INTERMEDIATES IN THE ASSEMBLY*

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Stable transfected baby hamster kidney (BHK) cells expressing human α, β, and γ fibrinogen chains together, in various combinations of any two, or individually, were established. Several types of subunit interactions were observed in the intracellular extracts of the transfected BHK cell lines as well as in Hep G2 cells. These included: 1) formation of αγ dimers linked by a disulfide bond (s), 2) formation of βγ dimers linked by a disulfide bond (s), 3) formation of αβγ half-molecules linked by disulfide bonds, and 4) formation of mature fibrinogen, which was also secreted into the cell culture medium. Analysis of the chain composition confirmed the stoichiometry of the αγ, βγ, and αβγ complexes. These data are consistent with the proposal that the αγ and βγ dimers as well as the αβγ half-molecules are intermediates in the assembly and biosynthesis of fibrinogen. In contrast, disulfide-linked aβ complexes were not found in transfected BHK cells or in Hep G2 cells, suggesting that the formation of disulfide bonds between these two chains most likely occurs when αβγ half-molecules are formed from αγ and/or βγ complexes and when αβγ half-molecules dimerize to generate the mature molecule. Dimers, trimers, and larger oligomers of each individual chain linked by disulfide bonds were also identified when each chain was expressed in the absence of the other two chains. Preferential formation of αγ and βγ complexes, rather than oligomers of individual chains, suggested that the oligomers were less likely to be intermediates in the assembly of fibrinogen. A model for fibrinogen assembly is presented based on these results.

Fibrinogen is a plasma glycoprotein that participates in the final phase of the blood coagulation cascade. The molecule consists of two sets of α, β, and γ polypeptide chains with molecular weights of 66,000, 52,000, and 46,500, respectively (McKee et al., 1996). The six chains, (αααγγγ), are linked by 29 disulfide bonds to form a trinodular structure as seen in the electron microscope (Hall and Slayter, 1959; Erickson et al., 1983). During the coagulation process, thrombin removes the amino-terminal fibrinopeptides A and B by minor proteolysis, converting fibrinogen to fibrin monomers. The fibrin monomers then polymerize to form an insoluble fibrin clot (Doolittle, 1973, 1975) that is cross-linked by factor XIIIa.

cDNA cloning has shown that the α, β, and γ chains of fibrinogen are encoded by three independent genes (Chung et al., 1983a, 1983b, 1990; Rixon et al., 1983; Kant et al., 1983) and are synthesized in liver hepatic parenchymal cells (Forman and Barnhart, 1964) as well as in cell cultures in Hep G2 cells (Knowles et al., 1980). The individual chains are processed, glycosylated, assembled, and eventually secreted into the circulating blood as mature fibrinogen molecules. It is generally agreed that the intracellular assembly of the three peptide chains into fibrinogen is a stepwise process involving a number of probable intermediates, but there are conflicting reports concerning both the intermediates and pathway of assembly. In one model (Yu et al., 1983, 1984, 1986), αβ and βγ dimers were proposed to be intermediates which, upon addition of a third chain, form the αβγ half-molecule and then mature fibrinogen. In other models (Doolittle, 1984; Hartwig and Danisehsfky, 1991), different combinations of fibrinogen chains including γαα, αγγ, and/or βγγ were proposed to be intermediates. Dimerization of αγγ and βγγ formed complicated intermediates from which the extra γ chains were then displaced to form the mature fibrinogen. In addition, a precursor-product relationship between these putative intermediates of fibrinogen biosynthesis was not established.

Human fibrinogen has been expressed in several types of transfected mammalian cells in culture including baby hamster kidney (BHK) cells (Farrell et al., 1989; Roy et al., 1991; Hartwig and Danisehsfky, 1991). The composition of the α, β, and γ chains of recombinant fibrinogen secreted into the culture medium by transfected BHK cells was essentially identical to that of plasma fibrinogen. Furthermore, the recombinant fibrinogen was functionally indistinguishable from plasma-derived fibrinogen in terms of its conversion to fibrin, polymerization, cross-linking, and platelet aggregation activity (Farrell et al., 1991, 1992a, 1992b). Recombinant fibrinogens in which the RGD sequences in the α chain were mutated were also fully active in platelet aggregation while those with a variant carboxyl-terminal end of the γ chain were inactive (Farrell et al., 1992a, 1992b).

In the present work, the intracellular interactions of the various chains of fibrinogen were examined by expressing each of the α, β, and γ chains individually or in various combinations in BHK cells. Complexes of αγγ, βγγ, and αβγ were identified and characterized both in transfected BHK cells and Hep G2 cells. From these data, a proposed mechanism for the assembly of fibrinogen has been presented.

MATERIALS AND METHODS

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from Promega and Boehringer Mannheim, Sequenase Kit from U. S. Biochemicals, cell culture media from Gibco and JHR Scientific, and fetal

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1 The abbreviations used are: BHK, baby hamster kidney; PAGE, polyacrylamide gel electrophoresis; BfP, binding protein.
bovine serum from Hyclone. L-[35S]Met and L-[35S]Cys (approximately, 1.1 Ci/mmol) were obtained from Amersham Corp. Protein-A Sepharose was from Sigma. Antibodies against human fibrinogen and binding protein, BiP, were from Accurate Chemical and Stressgene, respectively. Brefeldin A was from Epicentre Technologies. Chain-specific antisera against α, β, and γ chains of human fibrinogen were kindly provided by Dr. Edward Flow at Research Institute of Scripps Clinic, La Jolla, CA.

Construction of Expression Vectors—Mammalian expression vector pZem229 was used to express individually each of three cDNAs for construction and preparation of the expression vectors. Mammalian pZem229 to generate expression plasmids pAZem229 for the regions of the resulting plasmids were confirmed by dideoxy sequencing to ensure that no base substitution was introduced during the level of expression than pZem229 (Mulvihill et al., 1988). The cDNA for the α chain of human fibrinogen was released from plasmid pAG-1 (Farrell et al., 1991) by digestion with restriction enzyme BamHI and then directly inserted into the BamHI and Bgl II cloning sites of pZem97 to generate expression plasmid pAZem97 (Fig. 1).

Cell Culture and DNA Transfection—The BHK 2170 cell line (African Green Monkey Cercopithecus aethiops, ATCC) was maintained in Dulbecco’s modified Eagle’s medium (JRH Scientific), supplemented with 5% (v/v) fetal bovine serum (HyClone), and antibiotics (final concentration 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin, GIBCO). Hep G2 cells were maintained in minimum Eagle’s medium (GIBCO) with 10% fetal bovine serum and antibiotics (final concentration 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin, GIBCO). DNA transfections were carried out by a modified calcium phosphate precipitation procedure (Graham and van der Eb, 1973). In single plasmid transfections 25 μg of DNA was used while, in cotransfections, 25 μg of each plasmid was employed. Methotrexate (final concentration 1 μM) was then added to the culture media 24 h post-transfection. Resistant colonies were randomly selected 10 days later and their cell extracts and culture media subjected to Western blot analysis for the expression of fibrinogen chains or secreted fibrinogen. Producing cell lines were established and maintained in 1 μM methotrexate to further amplify the transfected genes.

Metabolic Labeling—BHK cells, 90% confluent, were labeled in Met- and Cys-free Dulbecco’s modified Eagle’s medium supplemented with 5% dialyzed fetal bovine serum, 100 μCi/ml each of [35S]Met and [35S]Cys, 18 h. Hep G2 cells, ~80% confluence, were labeled in Met- and Cys-free minimal Eagle’s medium, supplemented with 10% dialyzed serum, 200 μCi/ml of [35S]Met and 200 μCi/ml of [35S] Cys for 18 h. After labeling, the culture media were collected and a protease inhibitor mixture was added (final concentration 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml peptatin, 5 mM 4-aminocaproic acid, 5 mM EDTA, and 50 mM iodoacetamide). Cells were then washed with phosphate-buffered saline three times and lysed, for 2-10 min on ice, with lysis buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 300 mM sucrose, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.2% SDS with the protease inhibitors described above. The media and cell lysates were centrifuged to remove cell debris and stored at -70 °C.

Immunoprecipitation, Electrophoresis, and Western Blot Analysis—All immunoprecipitations were carried out at 4 °C. The media and cell lysates were preadsorbed with normal rabbit serum for 1 h followed by the incubation with Protein A-Sepharose for an additional 1 h, and the adsorbed material was removed by centrifugation. Rabbit antibodies directed against human fibrinogen or individual chains were added to the remaining supernatant fractions and incubated with gentle mixing for 1 h. Protein A-Sepharose was then added, and the supernatant was incubated for an additional 1 h. The beads were then collected by centrifugation for 1 min and washed successively with lysis buffer, phosphate-buffered saline containing 500 mM NaCl, and 1% Nonidet P-40 and 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1 mM EDTA, respectively. The immunoprecipitated proteins were released from Protein A-Sepharose by boiling for 5 min in 1.5 x SDS-PAGE loading buffer with or without 5% 2-mercaptoethanol.

Electrophoresis including one and two-dimensional non-reducing/reducing SDS-PAGE was performed according to Laemmli (1970) and Davidson et al. (1977), respectively. Gels for autoradiography were soaked in Amplify (Amersham Corp.) prior to drying. In some cases radioactive protein bands were excised from the two-dimensional gels, solubilized at 85 °C for 3 h with occasional shaking in 18% perchloric acid containing 18% hydrogen peroxide, and the radioactivity quantitated by a liquid scintillation counter. The specific radioactivities for each of the three chains were calculated by dividing the radioactivity by the total number of methionine and cysteine residues in each chain (Henschel and Lottspeich, 1977; Lottspeich and Henschel, 1977; Doolittle et al., 1979; Henschel et al., 1979; Watt et al., 1979).

Western blot transfers were carried out as described (Towbin et al., 1979). Immunoblotting was performed with either rabbit anti serum against human fibrinogen or monoclonal antibody against human heat shock protein 70 as the first antibody, and protein was detected with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Purification of Amino Acid Sequence Analyses of the complex was isolated from a cell lysate using an antibody affinity column followed by SDS-PAGE. An IgG fraction from rabbit anti serum against human fibrinogen (Accurate Chemicals) was coupled

**Fig. 1. Expression vectors for the α, β, and γ chains of fibrinogen.** A cDNA coding for each of the three chains was inserted into the cloning site of plasmid pZem229 to obtain expression vector pAZem229 for the α chain, pBZem229 for the β chain, and pGZem229 for the γ chain, respectively. The α-cDNA was also inserted into the cloning site of plasmid pZem97 to obtain low level expression vector pAzem97 for the α chain. The arrows indicate direction of transcription, SV40, SV40 early promoter/enhancer, SV40-1, SV40 terminator; bGH, human growth hormone terminator; MT-1, mouse metallothionine I promoter; DHFR, mouse dihydrofolate reductase cDNA.
to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instruction. All subsequent procedures were carried out at 4 °C. Cells expressing the \( \alpha \gamma \) complex (AG 1302, Table I) were grown in 150-mm plates. Cells from 50 such plates were lysed in 50 ml of lysis buffer for 30 min. An equal volume of 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and 1 mM EDTA was then added. The suspension was centrifuged at 10,000 \( \times g \) for 30 min. The supernatant was added to 20 ml of antibody-coupled resin and mixed overnight. After centrifugation at 2,000 \( \times g \) for 10 min, the supernatant was discarded, and the resin was packed into a column 15 × 25 mm in size. The column was washed with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100) followed by buffer B (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA) and then eluted with 200 mM glycine, pH 2.8, containing 500 mM NaCl. The fractions (0.5 ml) were immediately neutralized with 200 \( \mu l \) of 2 M Tris base and precipitated with methanol/chloroform (Wessel and Flugge, 1984). The precipitate was dissolved in SDS-PAGE sample buffer (without 2-mercaptoethanol) by boiling for 5 min. The immunopurified proteins were resolved by 7.5% SDS-PAGE, transferred to Immobilon membrane (Millipore), and stained with Coomassie Blue. The stained band with an apparent molecular weight of 154,000 was excised from the Immobilon sheet and subjected to NH\(_2\)-terminal amino acid sequence analysis in an Applied Biosystems 477A protein sequencer equipped with an on-line phenylthiohydantoin analyzer (model 120A). Repetitive yields of 94% were routinely obtained.

RESULTS

Stable Transfected BHK Cell Lines Expressing Fibrinogen Chains—Stable BHK cell lines (Table I) were obtained by transfecting BHK 570 cells with various expression plasmids and selecting for high-producing methotrexate-resistant colonies. Expression of fibrinogen and fibrinogen chains by these cell lines was established by Western blot analysis and metabolic labeling. Intracellular extracts were then examined and compared with similar extracts from Hep G2 cells. In each of the cell lines, the expressed fibrinogen chains comigrated with those isolated from Hep G2 cells in reduced SDS-PAGE (data not shown). The apparent mass of the complexes as estimated by SDS-PAGE in the absence of a reducing agent, however, was usually larger than that calculated from amino acid analysis. The cell lines used in this study and their characteristics are summarized in Table I.

Complex Formation in Cell Lines Coexpressing Two of the Three Fibrinogen Chains—The intracellular fibrinogen chains being expressed were analyzed by immunoprecipitation of radiolabeled cell lysates, using human fibrinogen-specific antisera and Protein A-Sepharose, followed by two-dimensional SDS-PAGE. The two-dimensional gel system made it possible to readily determine the chain composition of individual complexes. In these experiments, the radiolabeled complexes were initially separated by SDS-PAGE in the absence of a reducing agent and then in a second dimension in the presence of 2-mercaptoethanol. The component chains of each radioactive band in the non-reduced gel were then identified.

When \( \beta \) and \( \gamma \) chains were coexpressed, as in cell line BG4, several bands were observed in the non-reduced gel (Fig. 2). The major band migrated with an apparent mass of 134 kDa. The free \( \gamma \) chain (43 kDa) as well as a band of 79 kDa corresponding to BiP (see below) were also observed. In the reduced gel (Fig. 2), the 134 kDa band was found to contain both \( \beta \) and \( \gamma \) chains in about equal amounts. The very small amounts of higher molecular mass complexes also contained both \( \beta \) and \( \gamma \) chains. One complex (apparent mass of 102 kDa), also present in trace amounts, contained only \( \gamma \) chains. These results indicated that coexpression of \( \beta \) and \( \gamma \) chains leads mainly to the formation of a \( \beta \gamma \) dimer held together by disulfide bond(s) as well as free \( \gamma \) chains. Also, the \( \beta \gamma \) dimers as well as the free \( \gamma \) chains were apparently associated with BiP which coprecipitated with the \( \beta \gamma \) or \( \gamma \) chain-antibody complexes.

When the \( \alpha \) and \( \gamma \) chains were coexpressed, as in cell line AG1302, a number of radiolabeled bands were observed in the non-reduced gel (Fig. 3). The major complex migrated with an apparent mass of 154 kDa. There were also lesser amounts of free \( \alpha \) and \( \gamma \) chains, as well as three complexes with apparent masses greater than 250 kDa. In the reduced gel, the 154-kDa complex migrated as \( \alpha \) and \( \gamma \) chains present in approximately equal amounts. The three large complexes also contained \( \alpha \) and \( \gamma \) chains. A strong band was also noted for BiP. These results indicated that coexpression of \( \alpha \) and \( \gamma \) chains leads primarily to the formation of an \( \alpha \gamma \) complex held together by disulfide bond(s).

| Table I |
| Characteristics of the stable transfected BHK cell lines expressing human fibrinogen chains |

BHK 570 cells were transfected with expression plasmids for each of the three chains of fibrinogen, selected with various concentrations of methotrexate (MTX), and characterized as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Transfected plasmids</th>
<th>MTX</th>
<th>Fibrinogen chains expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A104</td>
<td>pA7em229</td>
<td>1</td>
<td>( \alpha )</td>
</tr>
<tr>
<td>B1209</td>
<td>pB7em229</td>
<td>20</td>
<td>( \beta )</td>
</tr>
<tr>
<td>G103</td>
<td>pG7em229</td>
<td>1</td>
<td>( \gamma )</td>
</tr>
<tr>
<td>A12123</td>
<td>pA7em297, pB7em229</td>
<td>10</td>
<td>( \alpha, \beta )</td>
</tr>
<tr>
<td>BG4</td>
<td>pB7em229, pG7em229</td>
<td>1</td>
<td>( \beta, \gamma )</td>
</tr>
<tr>
<td>AG1302</td>
<td>pA7em229, pG7em229</td>
<td>1</td>
<td>( \alpha, \gamma )</td>
</tr>
</tbody>
</table>

FIG. 2. Coexpression of \( \beta \) and \( \gamma \) chains. Transfected BHK cell line BG4 (Table I) was plated onto 35-mm dishes, and 24 h later, the cells were labeled for 18 h with 100 \( \mu Ci/ml \) of [\( ^35 \)S]Met and [\( ^35 \)S]Cys in the presence of 5% dialyzed fetal bovine serum. Cell lysates were immunoprecipitated with anti-fibrinogen antibody and the immunoprecipitate analyzed by two-dimensional SDS-PAGE as described under "Materials and Methods." The autoradiogram of the first-dimensional gel strip is shown on top of the second-dimensional gel so that the disulfide-linked complexes on the non-reduced gel were aligned with their component chains on the reduced gel. The arrows indicate the direction of electrophoresis. The molecular masses of the \( ^{13} \)C-labeled protein standards are indicated.
When α and β chains were coexpressed, as in cell line AB1223, a large number of high molecular mass bands as well as free α and β chains were observed in the non-reduced gel (Fig. 4A). The reduced gel showed that these bands contained primarily β chains with trace amounts of α chains. It was not possible, however, to determine whether any trace amounts of an αβ complex were present. Since individual chains were also capable of forming oligomers (see below), it was possible that the α and β chains seen in the reduced gel were from α and β oligomers. To address this possibility, a β chain-specific antibody was used in immunoprecipitation prior to the two-dimensional gel analysis. These results showed that no α chain was coimmunoprecipitated by this antibody (Fig. 4B). These results demonstrated that coexpression of α and β chains in BHK cells did not lead to the formation of detectable amounts of an αβ dimer. This was in contrast to the formation of αγ and βγ dimers, as described above. These experiments also suggested that the complexes observed in the non-reduced gel (Fig. 4A) originated separately from oligomers of α or β chains.

Complex Formation in Cell Lines Expressing Only the α or β or γ Chains of Fibrinogen—As shown in Fig. 5, the monomeric form of the α, β, and γ chains of fibrinogen were generated in addition to several higher molecular mass complexes when BHK cells were expressing only one fibrinogen chain. The compositions of the high molecular mass complexes were then determined by two-dimensional SDS-PAGE analysis, as previously described. These experiments demonstrated that the high molecular mass complexes were composed of dimers, trimers, and larger polymers of the corresponding fibrinogen chains (data not shown). The apparent masses and the possible oligomeric states of these complexes are summarized in Table II. The heterogeneity observed for some of these oligomeric forms of fibrinogen chains probably reflects in part a different degree of post-translational modification as well as some degradation by intracellular proteases.

A band with an apparent mass of 79 kDa was also present in the immunoprecipitates from all BHK cell lines expressing fibrinogen chains as well as from Hep G2 cells (Figs. 2–6). To identify this protein, the immunoprecipitates obtained with antibody against fibrinogen were subjected to Western blot analysis followed by immunostaining with a monoclonal antibody, N27F3-4 (Stressgene), made against heat shock protein 70 (Kost et al., 1989). These results (data not shown) revealed that this protein was human heat shock protein 70, also known as the binding protein, BiP (Haas and Wabl, 1983; Rothman, 1989).

Formation of α, β, and γ Complexes in Hep G2 Cells—Intracellular fibrinogen and complexes of fibrinogen chains present in Hep G2 cells were also analyzed using the same approach as employed for the transfected BHK cells (Fig. 6). The fully assembled fibrinogen was readily resolved into α, β, and γ chains following reduction of the protein. Three other complexes were also observed, including one corresponding to
FIG. 5. Expression of individual α or β or γ chains in BHK cells. Transfected BHK cell lines A104 (α), B1229 (β), and G103 (γ) (Table I) were plated into 35-mm dishes, and 24 h later, they were labeled for 18 h with 100 μCi/ml of [35S]Met and [35S]Cys in the presence of 5% dialyzed fetal bovine serum. Cell lysates were immunoprecipitated with chain-specific antibodies. The immunoprecipitates were analyzed in a 7.5% SDS gel under non-reducing conditions. Based on their amino acid composition, the molecular mass of the complexes were then assigned based solely on gels and compared with those of the standards. The oligomeric complexes included the α- and β- and γ- chains. These same fibrinogen complexes, as well as intact fibrinogen, were also observed in the culture media of transfected BHK cells (data not shown). No βγ dimers or free β chains, however, were detected in the culture media. It seems unlikely that the αγ and αβγ complexes as well as fibrinogen present in the culture medium resulted from dead cells for the following reasons: 1) αγ dimers were detected in pulse-chase experiments (data not shown), and in these experiments cell death was minimal since cells were employed in their early growth phase and were washed extensively with the growth medium before chasing; 2) the relative amount of various partial complexes were different inside the cell and in the medium and some complexes, such as the β chain, and the βγ dimer as well as BiP were not detected in the media; and 3) secretion of fully assembled fibrinogen in Hep G2 cells was blocked by including in the culture medium 1 μg/ml brefeldin A, a fungal metabolite that inhibits protein secretion and intracellular transport in rat hepatocytes (Oda et al., 1987; Misumi et al., 1996; Orci et al., 1991) and this also blocked secretion of the partial complexes (data not shown).

FIG. 6. Formation of partial fibrinogen complexes in Hep G2 cells. Hep G2 cells were plated in 100-mm dishes and, 24 h later, they were radiolabeled with 200 μCi/ml [35S]Met and [35S]Cys in the presence of 10% dialyzed fetal bovine serum for 18 h. The cell lysate was immunoprecipitated and analyzed by two-dimensional SDS-PAGE as described in Fig. 2. Only the autoradiogram for the second-dimensional gel is shown.

FIG. 7. Secretion of partial fibrinogen complexes by Hep G2 cells. Experimental conditions were identical to those described in Fig. 6 except that the culture medium was immunoprecipitated and analyzed.

TABLE II
Apparent molecular mass of intracellular oligomers of α, β, and γ chains of human fibrinogen

<table>
<thead>
<tr>
<th>Fibrinogen chains (apparent molecular mass, kDa)</th>
<th>α</th>
<th>β</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>63</td>
<td>41/45</td>
<td>37/39</td>
</tr>
<tr>
<td>Dimer</td>
<td>129</td>
<td>118</td>
<td>95/102</td>
</tr>
<tr>
<td>Trimer</td>
<td>166</td>
<td>138</td>
<td>127</td>
</tr>
<tr>
<td>Tetramer</td>
<td>245</td>
<td>235</td>
<td>190</td>
</tr>
<tr>
<td>Higher order</td>
<td>280</td>
<td>236</td>
<td>225</td>
</tr>
<tr>
<td>Oligomers</td>
<td>&lt;340</td>
<td>&lt;340</td>
<td>&lt;340</td>
</tr>
</tbody>
</table>

αβγ half-molecules with an apparent mass of 260 kDa, a second corresponding to αγ dimers with an apparent mass of 154 kDa, and a third corresponding to βγ dimers with an apparent mass of 134 kDa as estimated in the non-reducing gels. No αβ bands were observed in extracts from the Hep G2 cells. These results were analogous to those found with the transfected BHK cells. The presence of these three partial complexes in Hep G2 cells is consistent with the proposal that they are intermediates in fibrinogen assembly and not merely artifacts of the transfected BHK cells.

Fully assembled fibrinogen, as well as partial complexes, were also detected in the culture medium of Hep G2 cells (Fig. 7). In these experiments, the culture medium from the radiolabeled Hep G2 cells was immunoprecipitated and subjected to two-dimensional gel analysis. The complexes included the αβγ half-molecule, the αγ dimer, as well as free α and γ chains. These same fibrinogen complexes, as well as intact fibrinogen, were also observed in the culture media of transfected BHK cells (data not shown). No βγ dimers or free β chains, however, were detected in the culture media. It seems unlikely that the αγ and αβγ complexes as well as fibrinogen present in the culture medium resulted from dead cells for the following reasons: 1) αγ dimers were detected in pulse-chase experiments (data not shown), and in these experiments cell death was minimal since cells were employed in their early growth phase and were washed extensively with the growth medium before chasing; 2) the relative amount of various partial complexes were different inside the cell and in the medium and some complexes, such as the β chain, and the βγ dimer as well as BiP were not detected in the media; and 3) secretion of fully assembled fibrinogen in Hep G2 cells was blocked by including in the culture medium 1 μg/ml brefeldin A, a fungal metabolite that inhibits protein secretion and intracellular transport in rat hepatocytes (Oda et al., 1987; Misumi et al., 1996; Orci et al., 1991) and this also blocked secretion of the partial complexes (data not shown).

**Stoichiometry of Partial Fibrinogen Complexes**—The stoichiometry of the αγ, βγ, and αβγ complexes was determined by measuring the ratio of specific radioactivity of the component chains after separation by two-dimensional SDS-PAGE. Partial fibrinogen complexes in both transfected BHK cells and Hep G2 cells as well as fully assembled fibrinogen in Hep G2 cells were radiolabeled with [35S]Cys and [35S]Met. 2 S. Huang, unpublished observation.
of identical specific activities (Table III). The ratio of $^{35}$S radioactivity in each chain of secreted mature fibrinogen served as a control. The 154 kDa band was composed of equal molar ratios of α and γ chains and thus represents the αγ dimer. Likewise, the 134 kDa band was a βγ dimer, and the 260 kDa band was the αβγ half-molecule. As expected, there was some discrepancy between the apparent molecular mass estimated on the non-reduced SDS-PAGE and the calculated molecular mass based on the stoichiometry determinations for the partial complexes. In the case of the 260-kDa αβγ complex, with 1:1 stoichiometry, calculated molecular mass for a half-molecule was 170 kDa (66 kDa for the α, 52 kDa for the β, and 47 kDa for the γ chain, respectively) which was smaller than that estimated by the non-reduced SDS-PAGE. Similarly in the cases of 154-kDa αγ and 134-kDa βγ complexes, with 1:1 stoichiometry of the component chains they could be either αγ and βγ, or αγγ and βγγ, respectively. The latter forms were unlikely since both these complexes migrated faster than the half-molecule. The calculated molecular masses for αγ and βγ complexes were 113 and 99 kDa, respectively, both of which were smaller than what appeared on the non-reduced SDS-PAGE.

While determining the stoichiometry of the βγ complex in BG4 cells, some discrepancy was also observed when different antibodies were used for immunoprecipitation. With β chain-specific rabbit antisera, the ratio of the β chain to the γ chain was 1 to 1 (Table III). However, when a rabbit antisera which recognizes all three chains as well as fibrinogen (Accurate Chemicals) was used, a higher ratio of the γ chain was obtained (data not shown). The higher ratio of the γ chain obtained with anti-fibrinogen antibody was most likely due to the presence of some γ trimers that migrated very close to the 134-kDa βγ complex.

The identity and stoichiometry of the αγ complex was also indicated by preliminary amino acid sequence analysis. When the αγ dimer purified from the cell lysate of cell line AG1302 was subjected to amino acid sequence analysis, 2 amino acids were released at each cycle and these residues corresponded to those present in the α and γ chains of human fibrinogen (data not shown). This confirmed the identities of the component chains in the complex and also suggested a 1 to 1 molar ratio of α and γ chains.

**DISCUSSION**

The assembly of subunits of multimeric proteins is a complex problem since it usually involves the association of individual polypeptide chains in a specific manner. In the case of fibrinogen, subunit assembly is further complicated by the fact that fibrinogen is comprised of three different chains linked by disulfide bonds. In the present studies, a eukaryotic expression system has been employed for fibrinogen biosynthesis to test for subunit interactions that may occur during the assembly of the molecule. The results suggested three principle types of early subunit interactions among the fibrinogen chains that generated complexes linked by disulfide bonds. They were: 1) interactions of β and γ chains, 2) interaction of α and γ chains, and 3) interaction of different chains. In cell lines coexpressing α and γ chains, or β and γ chains, only the chains which are in relative excess form oligomers of individual chains, while the majority of the chains were incorporated into αγ or βγ complexes. These data suggested that the interactions between α and γ chains and between β and γ chains were predominant over that of identical chains when fibrinogen chains were coexpressed (Fig. 8). This suggested that oligomers of each of the three chains of fibrinogen were unlikely to be direct intermediates in fibrinogen assembly. The inability of α and β chains to form a disulfide-linked complex was somewhat unexpected, since in fibrinogen there are three disulfide bonds linking these two chains. Two occur in the disulfide rings and one in the NH₂-terminal region (Blombäck et al., 1976; Henschen, 1978). The inability of α and β chains to interact initially to form a disulfide-linked partial complex implies that the interactions between these two chains are a later event in the assembly of fibrinogen. Thus, the initial steps in fibrinogen assembly appeared to be the formation of either an αγ or βγ dimer or both (Fig. 8). The addition of a third chain to the αγ or βγ complex can give rise to an αβγ half-molecule which can then dimerize to form fibrinogen.

Similar fibrinogen complexes were also present in Hep G2

### Table III

**Ratios of the specific radioactivity of the component chains in fibrinogen and some of the partial complexes**

Transfected cells were radiolabeled in the presence of 5% dialyzed fetal bovine serum and Hep G2 cells in 10% dialyzed fetal bovine serum. Fibrinogen-related proteins were isolated by immunoprecipitation, and the radioactivity in each of the component chains of the partial complexes and fully assembled fibrinogen was determined after excision of the protein spots from two-dimensional non-reducing/reducing gels as described under "Materials and Methods." The specific radioactivity is expressed as counts/minute/total number of methionine and cysteine residues in a given chain. The ratio of the component chains in a given complex is then expressed using the specific radioactivity of the β chain as 1 for all the complexes except αγ complexes, for which that of the α chain is used as 1. All data are mean ± S.D. Fg, fibrinogen; n, number of experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Complexes</th>
<th>Molecular mass</th>
<th>Specificity of immunoprecipitating antibody</th>
<th>Ratio of component chains</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG1302</td>
<td>α, γ</td>
<td>154 kDa</td>
<td>Fg, α chain</td>
<td>1.0 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>BG4</td>
<td>β, γ</td>
<td>134 kDa</td>
<td>Fg, β chain</td>
<td>1.3 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>Hep G2 (media)</td>
<td>α, β, γ</td>
<td>260 kDa</td>
<td>Fg, α chain</td>
<td>1.1 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td>Hep G2 (lysate)</td>
<td>α, β, γ</td>
<td>260 kDa</td>
<td>Fg, β chain</td>
<td>1.2 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>α, γ</td>
<td>154 kDa</td>
<td>Fg, α chain</td>
<td>1.2 ± 0.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>β, γ</td>
<td>134 kDa</td>
<td>Fg, β chain</td>
<td>1.1 ± 0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Estimated molecular mass from the relative mobilities on SDS-PAGE.
FIG. 8. Model for assembly of human fibrinogen. The solid lines represent the dimerizing disulfide bonds, and the circles represent the disulfide rings.

cells and included the $\beta\gamma$ dimer, the $\alpha\gamma$ dimer, and the $\alpha\beta\gamma$ half-molecule. The presence of these complexes and the lack of an $\alpha\beta$ dimer in Hep G2 cells was consistent with the results observed in transfected BHK cells.

The present data differ somewhat from reports by other laboratories. The presence of an $\alpha\beta$ complex was reported in both Hep G2 and transfected COS-1 cells (Yu et al., 1984; Roy et al., 1991). This was in contrast to the present investigation where a variety of partial fibrinogen complexes including oligomers of each of the three chains were observed. However, no $\alpha\beta$ complexes were identified. Overlap in the apparent sizes of these complexes might explain the discrepancy in regard to the $\alpha\beta$ complex. Partial complexes with similar molecular weight and chain composition have been reported (Yu et al., 1984; Hartwig and Danishefsky, 1991), but the stoichiometry of the component chains was not determined. It is also very evident that the migration of fibrinogen polypeptide complexes linked by disulfide bonds on non-reduced SDS-PAGE does not correlate with the mass of the polypeptides. Therefore, an analysis of the composition and stoichiometry of the component chains was necessary in identifying partial fibrinogen complexes that may be intermediates in fibrinogen assembly. The absence of heterotrimeric complexes with compositions of $\alpha\gamma_2$ and $\beta\gamma_2$ was inconsistent with sequential chain displacement models in which a homotrimer (e.g. $\gamma_3$) was initially formed, followed by stepwise displacement of the other two chains (Doolittle, 1984; Hartwig and Danishefsky, 1991).

Site-specific mutagenesis studies have indicated that the cysteine residues at the NH$_2$ termini of fibrinogen chains, which form disulfide bonds connecting two half-molecules of fibrinogen, are not involved in the formation of $\alpha\gamma$, $\beta\gamma$, and $\alpha\beta\gamma$ complexes (Huang et al., 1992, 1993). This was demonstrated by the fact that mutating these cysteine residues to serine did not affect the formation of $\alpha\gamma$, $\beta\gamma$, and $\alpha\beta\gamma$ complexes. These mutations, however, readily blocked the formation of fibrinogen from the $\alpha\beta\gamma$ half-molecules. Thus, the formation of disulfide bonds joining the $\alpha\beta\gamma$ half-molecules appears to be the last step in fibrinogen assembly. Therefore, it is reasonable to postulate the order in which fibrinogen assembly proceeds: 1) formation of $\alpha\gamma$ and $\beta\gamma$ complexes via the ring disulfide bonds; 2) formation of $\alpha\beta\gamma$ half-molecule via the ring disulfide bonds; and 3) formation of fibrinogen via the disulfide bonds generated in the amino-terminal portion of the $\alpha$, $\beta$, and $\gamma$ chains (Fig. 8).

The requirement for cellular components in fibrinogen assembly is not known. BiP has been shown to be associated with intracellular fibrinogen chains and complexes in BHK and HepG2 cells. This suggests that association with BiP is an intrinsic property of fibrinogen chains. So far fibrinogen has been assembled from its component chains in two types of heterologous cells, BHK cells (Farrell et al., 1989, 1991) and COS-1 cells (Roy et al., 1991; Hartwig and Danishefsky, 1991). Thus, assembly of fibrinogen does not seem to need hepatocyte-specific machinery, and thus a general secretory mechanism seems to be sufficient to direct the assembly of fibrinogen. The information for assembly appears to be inherent in the fibrinogen chains themselves. The present results are consistent with this postulation and further suggest that formation of the half-molecule linked by disulfide rings follows preferred sequences of chain addition. The requirement of certain peptide sequences for assembly of multimeric
proteins has been demonstrated in the case of procollagen (Bornstein, 1974). Each of the component chains of procollagen is synthesized as large precursor with additional sequences at both NH₂-terminal and COOH-terminal ends. These additional sequences are required for the assembly of procollagen. Further study is needed to understand the relationship between amino acid sequence and assembly of fibrinogen.

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REFERENCES