The Role of $\alpha\gamma$ and $\beta\gamma$ Complexes in the Assembly of Human Fibrinogen*

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The role of $\alpha\gamma$ and $\beta\gamma$ dimers as intermediates in the assembly of fibrinogen was examined in cell fusion experiments using stably transfected baby hamster kidney cell lines expressing one or combinations of fibrinogen chains. Fibrinogen was readily formed and secreted into the culture media when cells co-expressing $\beta$ and $\gamma$ chains and generating $\beta\gamma$ complexes were fused with cells expressing only the $\alpha$ chain. Likewise, when cells co-expressing $\alpha$ and $\gamma$ chains and generating $\alpha\gamma$ complexes were fused with cells expressing only the $\beta$ chain, fibrinogen was also formed and secreted. The relative amounts of $\alpha\gamma$ or $\beta\gamma$ intermediates observed during fibrinogen biosynthesis were determined by the levels of the component chains; i.e. when the $\beta$ chain was limiting, the $\alpha\gamma$ dimer was the predominant intermediate; likewise, when the $\alpha$ chain was limiting, the $\beta\gamma$ complex was the predominant intermediate. The incorporation of preformed $\alpha\gamma$ and $\beta\gamma$ complexes into secreted fibrinogen did not require concurrent protein synthesis, as shown by experiments employing cycloheximide. These data strongly support the role of $\alpha\gamma$ and $\beta\gamma$ complexes as functional intermediates in the assembly of fibrinogen.

Human fibrinogen is a large soluble plasma protein that plays a critical role in protecting the vascular network against the loss of blood following tissue injury (Hantgan, 1987). Fibrinogen (MW 340,000) is composed of two sets of three polypeptide chains including the $\alpha$ (MW 66,000), $\beta$ (MW 52,000), and $\gamma$ (MW 46,500) chains (McKee et al., 1986). The six chains, (a$\beta$g)$_3$, contain 29 disulfide bonds and form a complex trinodular structure (Hall and Slayter, 1959; Erickson and Fowler, 1983) linked by five disulfide bonds to form the intact fibrinogen molecule (Huang et al., 1993b). It has not been clearly established, however, whether both $\beta\gamma$ and $\alpha\gamma$ are functional intermediates in fibrinogen assembly, since no precursor-to-product relationship has been demonstrated.

Imbalances in the intracellular levels of the $\alpha$, $\beta$, and $\gamma$ chains have been observed in hepatocytes and hepatoma cells from several species. A common feature is an excess amount of $\gamma$ chain, but limited levels of either the $\beta$ chain, as in human hepatocytes in culture (Yu et al., 1983 and 1984) and the rabbit (Alving et al., 1982), or the $\alpha$ chain, as in rat (Hirose et al., 1988) and chicken (Plant and Grieninger, 1986). Unequal rates of synthesis and/or intracellular degradation may contribute to this imbalance. The effect of an imbalance of the three chains on fibrinogen synthesis and assembly is not known. In cultured chicken embryonic hepatocytes, adding serum to the culture medium restored the balance of fibrinogen chains and resulted in the salvage of those chains, which otherwise were targeted for degradation (Grieninger et al., 1984). However, the mechanism for this restoration has not been established.

In the present studies, the role of the $\alpha\gamma$ and $\beta\gamma$ dimers as intermediates in the assembly of fibrinogen was examined using a cell fusion system employing stably transfected baby hamster kidney (BHK) cells expressing one or two of the fibrinogen chains. The formation of the $\beta\gamma$ and $\alpha\gamma$ complexes was then examined with $\alpha$, $\beta$, or $\gamma$ chains being limiting. Intracellular levels of fibrinogen chains and the $\alpha\gamma$ and $\beta\gamma$ complexes in two fibrinogen-producing human hepatoma cell lines, Hep G2 (Knowles et al., 1980) and HuH-7 (Nakabayashi et al., 1982), were also measured. These data provided additional evidence for the formation of $\alpha\gamma$ and $\beta\gamma$ dimers, as well as $\alpha\beta$ half-molecules as intermediates in the assembly of fibrinogen from its three individual chains. Furthermore, the cellular level of individual chains determines whether the $\alpha\gamma$ or $\beta\gamma$ complex is the predominant intermediate.

MATERIALS AND METHODS

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from Promega and Boehringer Mannheim, and the Sequenase Kit from U. S. Biochemicals. Cell culture media were purchased from Life Technologies, Inc. and JRH Scientific, and fetal bovine serum from Hyclone. Polyethylene glycol 4000 (PEG-4000) was from Life Technologies, Inc. (35)SMet and (35)SCys (approximately 1.1 Ci/mmol) were obtained from Amersham. Protein A-Sepharose and cycloheximide were from Sigma. Antibodies against human fibrinogen were from Accurate Chemical.

Cell Lines—The Hep G2 human hepatoma cell line (Knowles et al., 1980) was kindly provided by Dr. Mulvihill at ZyMoGenetics, Inc., Seattle, WA. HuH-7 human hepatoma cell line (Nakabayashi et al., 1982) was kindly provided by Dr. Nakabayashi from the University of Calgary, Alberta, Canada. The establishment and characterization of stably transfected BHK cell lines expressing individual fibrinogen chains and intact fibrinogen have already been described (Huang et al., 1989).

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1 The abbreviations used are: BHK, baby hamster kidney; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis.

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Hep G2 cells and stably transduced BHK cell lines were maintained as described earlier (Huang et al., 1993a). HuH-7 cells were maintained in RPMI 1640 medium containing 1% fetal bovine serum, antibiotics (50 mg/ml penicillin, 50 mg/ml streptomycin, and 100 mg/ml neomycin, Life Technologies, Inc.), and supplements as described (Nakahayashi et al., 1982).

**Construction of Expression Vectors**—The mammalian expression vector pZem229 (Mulvihill et al., 1988) was used to construct high-level expression vectors for the α, β, and γ chains of human fibrinogen (Huang et al., 1993a). The mammalian expression vector pZem97 was used to construct low-level expression vectors for the α chain, pA279 (Huang et al., 1993a), and the γ chain, pZem97. The expression vector pBD-1 (Farrell et al., 1991) containing the β chain cDNA was used as a low-level expression vector for this chain. The cell line BG500 was established by transfecting pZem229 (Huang et al., 1993a) into the cell line B1209-5, in which the expression level of the β chain was increased by selection and amplification in 5 μM methotrexate. In BG500 cells, approximately equal amounts of β and γ chains were synthesized as estimated by Western blotting and metabolic labeling analyses.

**Metabolic Labeling and PEG-induced Cell Fusion**—BHK cells (90% confluence) were labeled in Met-free and Cys-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% dialyzed fetal bovine serum, 200 μCi/ml [35S]Met and [35S]Cys for the indicated time. In cell fusion experiments, labeled cells were washed three times with Dulbecco’s modified Eagle’s medium and incubated with 2 ml of PEG-4000 (50% in phosphate-buffered saline) for 1.5 min to induce cell fusion. Cells were then washed five times with Dulbecco’s modified Eagle’s medium after removal of PEG-4000 and further incubated in growth medium for varying amounts of time as indicated. In some experiments, cells were incubated in growth medium containing cycloheximide (20 μg/ml) for 1 h before fusion and further incubated in the same medium after fusion. Hep G2 cells (90% confluence) were labeled in Met-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), containing 5% dialyzed serum and 200 μCi/ml [35S]Met. HuH-7 cells were labeled in Met-free RPMI 1640 (BioWhittaker), containing 1% dialyzed serum, supplements, and 200 μCi/ml [35S]Met. Cell lysates were prepared as described previously (Huang et al., 1993a) and stored at −70 °C.

**Immunoprecipitation and Electrophoresis**—Immunoprecipitation was carried out with a rabbit antibody (IgG fraction) to human fibrinogen and Protein A-Sepharose as described previously (Huang et al., 1993a).

Electrophoresis including one- and two-dimensional non-reduced/reduced SDS-PAGE were performed according to Laemmli (1970) and Davidson et al. (1977), respectively. Gels for autoradiography were impregnated with Amplify (Amersham) prior to drying. The radioactivity in the gel bands was quantitated by a PhosphorImager (Molecular Dynamics). The specific activities for each of the three labeled chains were calculated by normalizing the radioactivity to the number of methionine and/or cysteine residues in each chain (Henschen and Lottspeich, 1977; Lottspeich and Henschen, 1977; Doolittle et al., 1979; Henschen et al., 1979; Watt et al., 1979).

**RESULTS AND DISCUSSION**

**Formation and Secretion of Fibrinogen in Fused BHK Cells Expressing Fibrinogen Chains**—To determine whether αγ and βγ complexes were essential intermediates in the assembly of fibrinogen, experiments were designed to introduce one or more of the three chains of fibrinogen into individual stable BHK cells followed by cell fusion in the presence of PEG (Davidson et al., 1976). These experiments were undertaken to test whether fused cells could synthesize αγ, βγ, and αβγ complexes as well as mature fibrinogen. The formation of βγ and αγ complexes was initially examined by the fusion of stably transfected cells expressing the β chain with cells expressing the γ chain (beta cell/gamma cell fusion). By this approach, each pair of BHK cells were co-cultured in a dish, labeled with [35S]Met and [35S]Cys, and then fused in the presence of PEG. The intracellular complexes present in the fused cells were analyzed by immunoprecipitation, using a human fibrinogen-specific antibody and Protein A-Sepharose, followed by two-dimensional SDS-PAGE. The βγ complex was readily formed in the beta cell/gamma cell fusion (data not shown). Likewise, the αγ complex was rapidly formed after the fusion of alpha cells and gamma cells. However, no αβ complex was formed after fusion of alpha cells with beta cells. These preliminary results demonstrated that cells synthesizing individual fibrinogen chains remain competent to assemble potential fibrinogen intermediates after fusion and can be used to follow the precursor-to-product relationship in the assembly process. Since the fusion of cell mixtures is random, various combinations are likely to occur. Consequently, not all the labeled precursors are expected to be converted to assembled products in the fused cells.

Experiments were then carried out in which the α chain was introduced into BHK cells synthesizing and accumulating the βγ complex, and in which the β chain was introduced into cells synthesizing the αγ complex. In these experiments, two pairs of stably transfected BHK cell lines were used. The first pair included a cell line (BG500) which expressed β and γ chains generating βγ complexes, and a cell line (A104) that expressed only the α chain (Huang et al., 1993a). The second pair included a cell line (AG1302) that expressed α and γ chains generating αγ complexes and a cell line (B1209) that expressed only the β chain (Huang et al., 1993a). When the cells expressing the β and γ chains were fused with the cells expressing only the α chain (beta, gamma cell/alpha cell fusion), fully assembled intracellular fibrinogen was observed (Fig. 1A, lane 4). Very little or no fibrinogen was identified in the control or non-fused cells (lane 3). The assembled fibrinogen was also secreted into the culture medium (lane 2) in contrast to the control (lane 1). Two-dimensional, non-reduced/reduced SDS-PAGE showed that fibrinogen formed in the fused cells contained α, β, and γ.
The time course of intracellular fibrinogen formation in the fused cells was determined by Western blot analysis of cell lysates using an affinity purified antibody to human fibrinogen. As shown in Fig. 3, fully assembled fibrinogen was detected in both types of fused cells, including the alpha,gamma cell/alpha cell fusion and the alpha,gamma cell/beta cell fusion. Assembly was detectable within 30 min after PEG-induced fusion. As the post-fusion incubation time increased, the amount of fibrinogen increased.

In some experiments, (beta,gamma cell/alpha cell fusion), the beta chain, fibrinogen, as well as alpha chain dimers were formed in the fused cells (Fig. 1B). In these experiments, alpha dimers were generated in the alpha chain cell line. In other fusion experiments (alpha,gamma cell/beta cell fusion), the alpha and beta complexes, as well as alpha and beta complexes, were formed in the fused cells (Fig. 2B). The beta complex was generated by the interaction of beta chains in the beta chain cell line with the free excess beta chains in the alpha,gamma cell line. The presence of the beta complex in these fused cells suggested the possibility that fibrinogen may have been synthesized via the newly formed beta complex, as well as the pre-formed alpha complex.

**Effect of Cycloheximide on Fibrinogen Formation in Fused BHK Cells**—The effect of cycloheximide on the synthesis of fibrinogen was tested by measuring the incorporation of [35S]Met and [35S]Cys into fibrinogen chains after exposure to 20 μg/ml cycloheximide for 1 h. The intracellular fibrinogen chains were then immunoprecipitated and analyzed by reduced SDS-PAGE. The results indicated that the synthesis of fibrinogen was completely inhibited after incubation for 1 h with cycloheximide, as shown by the absence of any radioactive fibrinogen bands on the autoradiogram (Fig. 4).

To test whether pre-existing alpha and beta complexes were functional intermediates in the assembly process, cells were cocultured and prelabeled with [35S]Met and [35S]Cys, followed by incubation for 1 h with media containing cycloheximide to stop further protein synthesis. The cells were then fused with PEG and examined for intracellular fibrinogen. In order to avoid the complications of an excess of gamma chains in the assembly process, BHK cell lines BG1205 and AG105 were used in these fusion experiments. In the BG1205 cells, the beta chain was in considerable excess of the alpha chain and no detectable free alpha chain was present, as shown by the absence of a monomeric alpha chain on the autoradiogram (Fig. 5, lane 1). Similarly, in AG105 cells, the alpha chain was in considerable excess of the alpha chain. Consequently, there was no detectable amount of free intracellular alpha chain present as indicated by the absence of monomeric alpha chain on the autoradiogram (Fig. 5, lane 4).

When cell fusion experiments in these cells were carried out after inhibition of protein synthesis by cycloheximide, fibrinogen was formed in both the beta, gamma cell/alpha cell fusion (Fig. 5, lane 2), as well as the alpha, gamma cell/alpha cell fusion (Fig. 5, lane 4). Fibrinogen assembly did not occur in the control experiments (lanes 1 and 3). These data strongly suggest that fibrinogen was assembled from the pre-existing beta complex and the alpha chain in the beta, gamma cell/alpha cell fusion or from the pre-existing alpha complex and beta chain in the alpha, gamma cell/ beta cell fusion. These results support the conclusion that pre-formed beta and alpha complexes as well as individual alpha and beta chains were intermediates in fibrinogen assembly. Moreover, fibrinogen assembly did not require de novo protein synthesis.

**Effect of Imbalance of Fibrinogen Chains on Formation of Beta and Alpha Complexes in Transfected BHK Cells**—To examine further the effect of imbalance of fibrinogen chains on the formation of beta and alpha complexes, BHK cell lines expressing all three chains of fibrinogen, but only limiting amounts of the alpha chain (cell line FAl), the beta chain (cell line Fb), or the gamma chain (cell line FG), were established. Two promoters were used to express...
fibrinogen chains in these cell lines, including the wild-type metallothionein-1 promoter and a modified version. The expression level of the modified metallothionein-1 promoter was 10–20-fold higher than the wild-type metallothionein-1 promoter (Mulvihill et al., 1988). The latter promoter was used to express the fibrinogen chain which was designed to be limiting.

In the cell line FAl, the α chain was present in limiting amounts (Fig. 6A, lane 1). In this cell line, the major intracellular intermediate was the βγ complex, while only a trace amount of the αγ complex was observed (Fig. 6B, lane 1). In the cell line FBl, the β chain was limited relative to the α and γ chains (Fig. 6A, lane 2). In this cell line, the major form of intermediate was the αγ complex and only a trace amount of the βγ complex was generated (Fig. 6B, lane 2). In cell line FGi, the γ chain was limited relative to the α and β chains (Fig. 6A, lane 3). In these experiments, α and β chains were present as α chain oligomers and β chain oligomers, respectively. Both the βγ and αγ complexes were present in trace amounts as determined both by one-dimensional (Fig. 6B, lane 3) and two-dimensional SDS-PAGE (data not shown). Fully assembled fibrinogen was produced by all three of these cell lines (Fig. 7B) and secreted into the culture media (data not shown). The chain compositions of fibrinogen and the βγ and αγ intermediates in these cells were confirmed by two-dimensional SDS-PAGE analyses (data not shown).

These results demonstrated that the amounts of βγ and αγ intermediates were determined by the relative levels of the β and α chains. When the α chain was limiting, the βγ complex was formed preferentially, whereas in the case of limited β chain, the αγ complex was formed preferentially. However, when the γ chain was limited relative to the β and γ chains, formation of both βγ and αγ complexes were greatly reduced due to the limited availability of the γ chain.

Relative Rate of Synthesis of Fibrinogen Chains and Formation of βγ and αγ Complexes in Hep G2 and HuH-7 Cells—The rate of synthesis of fibrinogen chains in both Hep G2 cells and HuH-7 cells were determined by measuring the rate of appearances of [35S]Met in fibrinogen chains during the course of continuous labeling. In Hep G2 cells, there were approximately equal rates of synthesis for the α and β chains and a much faster rate of synthesis for the γ chain (Fig. 7A). In HuH-7 cells, approximately equal amounts of the β and γ chains were observed and a much smaller amount of the α chain (Fig. 7B).

These results indicated that the γ chain was in excess compared to the other two chains in Hep G2 cells, whereas the α chain was limited compared to the other two chains in HuH-7 cells.

Intracellular fibrinogen and fibrinogen intermediates in Hep
G2 and HuH-7 cells were analyzed using the same approach as employed for the transfected BHK cells (Fig. 8). Both fully assembled fibrinogen and various intermediates including αβγ half-molecules, αγ, and βγ complexes were present in both cell lines. However, the relative amounts of αγ and βγ complexes differed in these two cell lines. In Hep G2 cells, the amount of the αγ complex was greater than that of the βγ complex (Fig. 8, top). In contrast, in HuH-7 cells, the amount of the αγ complex was less than that of the βγ complex (Fig. 8, bottom). These results clearly showed that the relative amount of αγ and βγ complexes in human hepatoma cells was determined by the relative amounts of the component fibrinogen chains. In HuH-7 cells, the α chain was limiting and the βγ complex was preferentially formed. This was similar to the BHK cell line FAi, which was limited in the α chain (Fig. 6, lane 1). In Hep G2 cells, there were approximately equal amounts of the α and β chains, and a large excess of the γ chain. Under these conditions, more of the αγ complex was formed than the βγ complex. This suggested that the rate of formation and/or rate of degradation of the αγ and βγ complexes also affects the relative amount of the two αγ and βγ intermediates. Two-dimensional gel electrophoresis also showed that in both the Hep G2 and HuH-7 cells, the extended α chain variant, αE (Fu et al., 1992) was expressed and incorporated into fibrinogen (Fig. 8, top and bottom).

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