Biosynthesis, assembly and secretion of fibrinogen in cultured rat hepatocytes

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The biosynthesis, assembly and secretion of fibrinogen were investigated in cultured rat hepatocytes which were incubated with [³⁵S]methionine. When initial rates of the synthesis of three fibrinogen subunits were compared, the A α -subunit was found to be synthesized significantly slower than the B β - and γ -subunits. Pulse-chase experiments revealed that the secreted fibrinogen contained different proportions of the newly synthesized subunits, depending upon the chase times. Radioactivity in the A α subunit, which initially had the highest level of the three, was rapidly decreased in parallel with the chase time. The γ -subunit had an increasing amount of the radioactivity in the secreted molecule during the chase periods, whereas that in the B β -subunit was gradually decreased at the later stages of chase. Analysis of intracellular components of fibrinogen confirmed that the nascent A α -subunit was most rapidly exhausted, and the γ -subunit occupied the largest proportion among the non-assembled subunits at later stages of chase. Taken together, these results suggest that the synthesis of A α -subunit, which has the lowest rate, could be the rate-limiting step in the production and secretion of fibrinogen in cultured rat hepatocytes, in contrast with what has been proposed for human and rabbit fibrinogen, namely that the synthesis of B β -subunit is the rate-limiting step. The results also indicate that there is a large intracellular pool of γ -subunit.

INTRODUCTION

Fibrinogen, a plasma glycoprotein synthesized in the liver, is a large dimeric molecule (M_r 340 000) composed of three non-identical polypeptide chains. The M_r values of these chains (A α , B β and γ) were estimated to be 64 000, 57 000 and 47 000 respectively (Blombäck, 1979). The two half-molecules of the dimer are held together by symmetrical disulphide bonds in the A α - and γ -chains and, in addition, interchain bridges occur within the half-molecule.

Translation of fibrinogen mRNA by cell-free systems (Chung *et al.*, 1980; Yu *et al.*, 1980; Nickerson & Fuller, 1981) and molecular-cloning studies (Chung *et al.*, 1981; Crabtree & Kant, 1981; Kant *et al.*, 1983) have established that the three polypeptide chains of fibrinogen are synthesized by separate mRNAs. Previous studies on the biosynthesis of fibrinogen indicate that the newly synthesized chains are assembled in the rough endoplasmic reticulum (Bouma *et al.*, 1975; Kudryk *et al.*, 1982). A detailed analysis of the time course of rabbit fibrinogen secretion demonstrated that the nascent chains are not all simultaneously assembled, but that the newly formed molecule first contains nascent B β -chains, followed by nearly simultaneous incorporation of A α - and γ -chains (Alving *et al.*, 1982). Since then, pulse-chase experiments with human hepatocarcinoma cells (Hep-G2) confirmed that the nascent B β -chain is first incorporated into intracellular dimeric fibrinogen, followed by the two other subunits (Yu *et al.*, 1983, 1984). Furthermore, these studies with Hep-G2 cells demonstrated that a number of precursor forms of fibrinogen and a pool of γ -chain exist intracellularly (Yu *et al.*, 1983, 1984), suggesting that all the three nascent

chains are not simultaneously assembled in a simple manner in the rough endoplasmic reticulum.

In the present paper we describe the biosynthesis, assembly and secretion of fibrinogen in a primary culture system of rat hepatocytes, demonstrating that the nascent A α -chain is first incorporated into the dimeric fibrinogen molecule, followed by sequential incorporation of the B β - and then the γ -chain, in contrast with the results so far reported for rabbit fibrinogen (Alving *et al.*, 1982) and human (Yu *et al.*, 1983, 1984).

MATERIALS AND METHODS

Materials

L-[³⁵S]Methionine (1130 Ci/mmol) and Protosol were purchased from New England Nuclear (Boston, MA, U.S.A.). Sephadex G-100 was from Pharmacia Fine Chemicals (Uppsala, Sweden); aprotinin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); heparin and collagenase were from Wako Junyaku (Osaka, Japan); Eagle's minimum essential medium (MEM) was from Nissui Seiyaku (Tokyo, Japan); leupeptin, pepstatin, antipain, chymostatin, elastatinal and phosphoramidon were from Protein Research Foundation (Osaka, Japan).

Preparation of rat fibrinogen and anti-(rat fibrinogen) antiserum

Plasma freshly prepared from turpentine-treated (48 h) rats was used for purification of fibrinogen as described by Mosher & Blout (1973), with some modifications. Sephadex G-100 chromatography was employed as the final step instead of DEAE-Sephadex

Abbreviation used: MEM, minimum essential medium.

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chromatography. Ethanol-precipitated material (about 15 mg of protein) was dissolved in 2 ml of 10 mM-Tris/HCl, pH 7.4, containing 1% (w/v) SDS, 5 mM-2-mercaptoethanol, 10 mM-EDTA and 0.1 M-NaCl, boiled for 3 min and applied to a Sephadex G-100 column (2.5 cm $\times$ 90 cm) equilibrated with the same buffer, but without 0.1% SDS. Fractions containing fibrinogen subunits, which were identified by SDS/polyacrylamide-gel electrophoresis, were pooled. Antibodies against the purified fibrinogen subunits were raised in rabbits as described previously (Ikehara *et al.*, 1977).

Hepatocyte culture

Hepatocytes were isolated from adult male Wistar rats, weighing 250–350 g, by the collagenase-perfusion method (Seglen, 1976). Isolated cells were cultured in Eagle's MEM as described previously (Oda *et al.*, 1983), except that heparin (100 units/ml) was added to the culture medium 6 h after the inoculation of cells to prevent the degradation of fibrinogen (Grieninger *et al.*, 1978). All the following experiments were performed using the cells after 24 h culture. Heparin was present throughout the culture periods and pulse-chase experiments.

Pulse-chase experiments

Cells (2×10^6 cells/dish) were preincubated at 37 °C for 1 h in Eagle's MEM lacking methionine. The cells were pulse-labelled with [35 S]methionine (100 μ Ci/1.5 ml per dish) for the indicated times, and chased in the fresh medium containing unlabelled methionine (Oda *et al.*, 1983). At the indicated times, the medium was collected and replaced by the fresh medium. The cells were washed twice with Dulbecco's phosphate-buffered saline, pH 7.4, and lysed in 0.5 ml of distilled water containing 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 and heparin (100 units/ml). The medium and cell lysates were centrifuged at 15000 *g* for 10 min, and each supernatant was used for immunoprecipitation of fibrinogen.

Immunoprecipitation

A portion (2 vol.) of 50 mM-Tris/HCl, pH 7.4, containing 1% Triton X-100, 150 mM-NaCl and 5 mM-EDTA, was added to each cell lysate and medium, followed by the addition of a mixture of proteinase inhibitors (leupeptin, antipain, chymostatin, elastatinal, phosphoramidon and aprotinin; final concns. 10 μ g/ml). 35 S-labelled fibrinogen in each sample was immunoprecipitated by incubating with both anti-fibrinogen antiserum (20–50 μ l) and carrier fibrinogen (10–20 μ g) at 37 °C for 1 h and then at 4 °C overnight. The immunoprecipitates were washed and dissolved for analysis by SDS/polyacrylamide-gel electrophoresis as described previously (Oda *et al.*, 1983).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970). Unless otherwise indicated, samples were analysed by electrophoresis on 8.5%-(w/v)-polyacrylamide gels under reducing conditions. In some experiments, intracellular forms of fibrinogen were subjected to electrophoresis on 7.5% polyacrylamide gels under non-reducing conditions. Samples applied were adjusted to contain the same radioactivity (3000 d.p.m./slot). After electrophoresis,

gels were fixed and subjected to fluorography as described previously (Bonner & Laskey, 1974; Oda *et al.*, 1983).

Determination of radioactivity in the fibrinogen subunits

After SDS/polyacrylamide-gel electrophoresis, the radioactive subunits detected by fluorography were excised from gels, solubilized in Protosol and counted for radioactivity by liquid-scintillation spectrometry as described previously (Oda *et al.*, 1978). Radioactivities of the individual subunits were normalized by dividing the radioactivity in each subunit by the relative proportions of L-methionine ($A\alpha$, 10; $B\beta$, 15; γ , 8) (Henshen *et al.*, 1983). In some pulse-chase experiments the normalized radioactivities in each subunit of the fibrinogen were expressed as percentages of the total values of three subunits obtained at each time point.

RESULTS

Rate of synthesis of fibrinogen subunits

The initial rate of synthesis of the three fibrinogen subunits was determined by incubating hepatocytes with [35 S]methionine for various times up to 15 min. During the incubation the newly synthesized fibrinogen was totally retained within the cells, since no labelled fibrinogen was detectable in the medium. At the indicated times, the cells were harvested and the cellular fibrinogen was isolated by immunoprecipitation. The immunoprecipitates were subjected to SDS/polyacrylamide gel electrophoresis to separate the three subunits, radioactivities of which were determined and normalized to the methionine content of each subunit (Fig. 1). The $B\beta$ - and γ -subunits showed a similar time course of their synthesis. The synthesis rate of the $A\alpha$ subunit, however, was significantly lower than those of the $B\beta$ - and γ -subunits. The results are in contrast with those obtained for human fibrinogen synthesized by Hep-G2 cells, in which the synthesis of $B\beta$ was much slower than that of the other subunits (Yu *et al.*, 1984).

Changes in the distribution of radioactivity in secreted fibrinogen

It would be of interest to know whether the difference in the synthesis rates of the subunits is reflected in the assembly of fibrinogen. We examined the distribution of radioactivity in fibrinogen that was fully assembled and secreted after pulse-labelling. At first, hepatocytes were pulse-labelled for 3 min with [35 S]methionine and chased for up to 5 h with unlabelled methionine (0.1 mM). At the indicated intervals, the medium was collected and the secreted fibrinogen was isolated by immunoprecipitation. Radioactive fibrinogen appeared in the medium about 30 min after the start of chase and continued to be secreted for the period examined (5 h), although the secretion rate markedly decreased at later periods of chase. Samples prepared from each immunoprecipitate were adjusted to have the same radioactivity (3000 d.p.m./slot) and were analysed by SDS/polyacrylamide-gel electrophoresis-fluorography (Fig. 2a), followed by determination of the radioactivity in each subunit (Fig. 2b). At early times of secretion the radioactivity in the $A\alpha$ -subunit was much higher than those in the $B\beta$ - and γ -subunits, but rapidly decreased with chase time. A contrasting time course was obtained for the radioactivities for both the $B\beta$ - and γ -subunits, which were

Fig. 1. Rate of synthesis of A α -, B β - and γ -subunits

Hepatocytes (2×10^6 cells/dish) were incubated with [35 S]methionine ($50 \mu\text{Ci/dish}$) for the indicated times. The radioactive intracellular forms of fibrinogen were isolated by immunoprecipitation and subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by fluorography. The radioactivity of the individual subunits was determined and normalized to the methionine content of each subunit as described in the Materials and methods section. Subunits: $\circ$, A α ; $\bullet$, B β ; $\triangle$, γ . Each point shows the mean value obtained from two experiments (with variation less than 20%).

initially low and increased with time. The results indicate that the secreted fibrinogen contains the newly synthesized subunits with different rates of assembly. The A α -subunit, which has the lowest rate of synthesis (Fig. 1), was more effectively incorporated into the protein than were the two other subunits (Fig. 2). The time-dependent decrease in the A α -subunit suggests that the ^{35}S -labelled A α -subunit was rapidly exhausted and replaced by the unlabelled subunit under the conditions with the short-pulse label (3 min).

Such a difference in the assembly rate was confirmed by another pulse-chase experiment (Fig. 3). In this case, in order to label all the intracellular forms of fibrinogen as completely as possible, the cells were incubated with [^{35}S]methionine for a much longer period (3 h), and then chased. As shown in Fig. 3(b), all the three subunits in fibrinogen secreted at early times (up to 40 min) of chase were found to have a similar normalized radioactivity, in contrast with the results obtained with the short-pulse label (Fig. 2b). Thereafter, radioactivities of the three subunits showed different time courses. The radioactivity in the A α -subunit rapidly declined, as expected. In addition, there appeared to be a clear difference between those of the B β - and γ -subunits, which had not been so clearly demonstrated in the short-pulse-chase experiment; the proportion of radioactivity in the B β -subunit also gradually decreased at later times after 2 h of chase, whereas that in the γ -subunit continued to increase

Fig. 2. Distribution of radioactivity in fibrinogen secreted after a short-pulse label (3 min)

Cells were pulse-labelled for 3 min with [^{35}S]methionine ($100 \mu\text{Ci/dish}$) and chased for various periods of time up to 5 h. At the indicated times the medium was collected and replaced with fresh medium. ^{35}S -labelled fibrinogen was isolated from each sample of medium by immunoprecipitation, and samples with the same radioactivity (3000 d.p.m./slot) were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by fluorography (a). Lane 1, radioactive fibrinogen secreted between 0 and 40 min of the chase period; lane 2, that secreted between 40 and 60 min; lane 3, that between 60 and 80 min; lane 4, that between 80 and 100 min; lane 5, that between 100 and 160 min; and lane 6, that between 160 and 300 min of the chase period. (b) Quantitative analysis of the labelled subunits in the secreted fibrinogen. The radioactivity in the individual subunits separated as shown in (a) was determined and normalized to the methionine content of each subunit as described in the Materials and methods section. Values represent percentages of each subunit, taking as 100% the total radioactivity of the three subunits at each time point. Subunits: $\circ$, A α ; $\bullet$, B β ; $\triangle$, γ . Each point indicates the mean value ($\pm \text{s.d.}$) obtained in three experiments.

during the chase periods (Fig. 3b). Taken together, these results (Figs. 2 and 3) suggest that there must be a difference in intracellular pools of the three subunits available for the fibrinogen assembly.

Fig. 3. Radioactive composition of fibrinogen secreted after a long-pulse label (3 h)

Cells were labelled for 3 h with [35 S]methionine (100 μ Ci/dish) and then chased. Other experimental details were the same as those in Fig. 2. (a) SDS/polyacrylamide gel electrophoresis-fluorography. Lane 1, radioactive fibrinogen secreted during the pulse period; lane 2, that secreted between 0 and 20 min of chase; lane 3, that between 20 and 40 min; lane 4, that between 40 and 60 min; lane 5, that between 1 and 2 h; lane 6, that between 2 and 3 h; lane 7, that between 3 and 4 h; and lane 8, that between 4 and 5 h of chase. (b) Quantitative analysis of the labelled subunits in the secreted fibrinogen. Subunits: $\circ$, A α ; $\bullet$, B β ; $\triangle$, γ . Each point indicates the mean value ($\pm$ S.D.) obtained in three experiments.

DISCUSSION

Fibrinogen subunits are independently translated by different mRNAs (Chung *et al.*, 1981; Crabtree & Kant, 1981), indicating that the synthesis of subunits and their assembly into the functional protein could be regulated at different control points: transcriptional, translational and post-translational. Crabtree & Kant (1982) reported that defibrination with Malayan-pit-viper (*Agkistrodon rhodostoma*) venom causes a rapid and substantial accumulation in rat liver of the three mRNAs for the A α -, B β - and γ -subunits of fibrinogen, and that, during this response, the kinetics and extent of the increase are nearly identical for each mRNA, resulting in an increase in fibrinogen synthesis. Such a co-ordinate regulation of the three mRNAs might favour the earlier proposal that

the assembly of fibrinogen occurs co-translationally and no pool of subunits exists in the cell (Bouma *et al.*, 1975; Fuller, 1983). Recent observations, however, indicate that all the three nascent subunits are not simultaneously assembled in a simple manner (Alving *et al.*, 1982; Yu *et al.*, 1983, 1984).

In the present study, with rat hepatocytes in primary culture, we demonstrated that all the three subunits of fibrinogen are not synthesized with the same rate (Fig. 1). Pulse-chase experiments revealed that the three nascent subunits labelled with [35 S]methionine have different turnover rates, as judged by their appearance in the secreted fibrinogen (Figs. 2 and 3). 35 S-labelled A α -subunit, which has the lowest rate of synthesis, was most rapidly incorporated into precursor forms and into the mature form in the cells (S. Hirose, K. Oda & Y. Ikehara, unpublished work), and the secreted fibrinogen contained the labelled A α -subunit with the most rapid turnover among the three subunits. In contrast, the labelled γ -subunit, though synthesized at a similar rate to the B β subunit, had the slowest turnover under the conditions examined. Taken together, these results indicate that there are different intracellular pools for the individual subunits; the largest pool for the γ -subunit, the smallest for the A α -subunit and intermediate for the B β -subunit. Thus, during the pulse and subsequent early periods of the chase time, the labelled nascent A α -subunit is immediately assembled with pre-existing non-labelled B β - and γ -subunits together with the labelled ones, resulting in its acquiring a higher radioactivity than those of the last two subunits in the assembled form. In parallel with the chase, the labelled A α -subunit is exhausted and replaced by the non-labelled newly synthesized chain, which is then assembled with the labelled B β - and γ -subunits still remaining in larger pools, leading to reciprocal changes in their radioactivities. Therefore the synthesis of A α -chains could be the rate-limiting step in the assembly and secretion of fibrinogen in rat hepatocytes under the present conditions. This is in contrast with previously obtained evidence that the production of B β -chains is the limiting factor for fibrinogen formation in rabbit liver (Alving *et al.*, 1982) and in human hepatocarcinoma cells (Yu *et al.*, 1983, 1984) and also with another finding, namely that no particular subunit is considered to be a rate-limiting factor in dog liver fibrinogen formation (Kudryk *et al.*, 1982). It is not clear at present whether these discrepancies are due to the species specificity or to different systems of analysis (i.e., *in vivo* or culture).

A large intracellular pool of free γ -subunit would raise the following questions. The first is: why is there a different in intracellular pools between the B β - and γ -subunits, despite there being no significant difference in their synthesis rates? Although we do not have our own experimental data, a possible explanation is that the difference might be caused by selective degradation of the B β -subunit compared with that of the others, in a manner similar to that suggested for chicken fibrinogen (Grieninger *et al.*, 1984). Another question is: why are the free γ -subunits accumulating in the cells not secreted? This implies that the hepatocytes have a transport mechanism that discriminates for secretion the assembled form of fibrinogen from the non-assembled fibrinogen subunits. Recent observations indicate that intracellular transport of some virus proteins also requires folding and assembly of polypeptide chains, which may be

controlled by an association with a particular component (Gething *et al.*, 1986), as has been suggested for the binding protein for the heavy chains of immunoglobulins (Hendershot *et al.*, 1987). No information about a mechanism involving such a component is available at present to explain fibrinogen assembly.

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