Assembly and Secretion of Recombinant Human Fibrinogen*

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Expression vectors containing full-length cDNAs for each of the human fibrinogen chains were constructed. COS-1 cells were transfected with single vectors, mixtures of two, or with all three vectors and stable cell lines selected. Cells transfected with single vectors, or with mixtures of any two vectors, expressed the appropriate fibrinogen chains but did not secrete them. COS cells transfected with three vectors expressed all of the chains and secreted fibrinogen. COS cells transfected with three vectors contained, intracellularly, a mixture of fibrinogen-related proteins. The four main intracellular products were nascent fibrinogen, an Aα-γ complex, free Aα chains, and free γ chains. This is a similar pattern to that noted in Hep G2 cells. The intracellular forms of fibrinogen were sensitive to endoglycosidase H, indicating that they reside in a pre-Golgi compartment. Secreted fibrinogen was endoglycosidase H-insensitive, suggesting that the secreted glycoprotein moieties were processed in the normal manner. When mixed with plasma fibrinogen, radio-labeled recombinant fibrinogen was incorporated into a thrombin-induced clot. These studies demonstrate that COS cells transfected with all three fibrinogen chain cDNAs are capable of assembling and secreting a functional fibrinogen molecule.

Fibrinogen is composed of three different polypeptides (Aα, Bβ, and γ), arranged as a dimer with each half-molecule containing a set of each of the chains. The two half-molecules are linked together by three disulfide bonds at the NH₂-terminal portions of the polypeptides. Two of the symmetrical bonds are between adjacent γ chains and one is between Aα chains. In addition a complex set of inter- and intrachain disulfide bonds (there are 29 disulfide bonds with no free sulfhydryl groups) are involved in maintaining proper structure (1-4).

Our studies are aimed at determining how this multichain protein is synthesized, assembled, and secreted. Hepatocytes are the principal site of synthesis and each of the component chains of fibrinogen is encoded by a separate gene (5-8). Previously we demonstrated that Hep G2 cells have surplus pools of Aα and γ chains that occur either as free chains or complexed to each other, primarily as an Aα-γ complex (9-11). Hep G2 cells maintain these surplus amounts of Aα and γ chains even when fibrinogen synthesis and secretion is stimulated by production of enhanced amounts of Bβ chain (12). Pulse-chase experiments demonstrated that chain assembly commences by the attachment of preformed Aα and γ chains to nascent Bβ chains. On completion of Bβ chain elongation, the Bβ-γ and Bβ-Aα complexes are released into the lumen of the rough endoplasmic reticulum and acquire the third chain to form half-molecules. The two half-molecules are then joined to form dimeric fibrinogen. Chain assembly occurs in the rough endoplasmic reticulum (13).

To obtain further information on the mechanisms which govern chain assembly we prepared a set of stable transfected COS-1 cells which express either the individual fibrinogen chains, mixtures of two of the chains, or all three chains and studied the assembly and secretion of fibrinogen.

EXPERIMENTAL PROCEDURES

Materials

L-[³⁵S]Methionine, approximately 1.1 Ci/mmol, was purchased from Du Pont-New England Nuclear, fetal calf serum from Hyclone, endoglycosidase H from Genzyme, geneticin from Sigma, restriction and modifying enzymes from Boehringer Mannheim, and T4 DNA ligase from New England Biolabs. Human fibrinogen (Imco, Stockholm), prepared as previously described (14), was stored at -70 °C as a stock solution of about 14 mg/ml in 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl and 1 mM EDTA. The fibrinogen present in this preparation was removed by affinity chromatography on gelatin-Sepharose (15). Fibrinogen concentration was measured spectrophotometrically in alkaline urea using ε228 = 16.5 at 282 nm. Human thrombin was obtained from the Department of Blood Coagulation Research, Karolinska Institutet, Stockholm, Sweden. Trypsin was from Bayer. Other reagents used have been described previously (9-12).

Cell Culture

COS-1 cells were maintained in Iscove's medium supplemented with 10% fetal calf serum (Hyclone) and 1% glutamine (16). Hep G2 cells were originally obtained in 1982 from Drs. Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia, PA; they were maintained in Eagle's minimal essential medium containing 10% fetal calf serum, 15 mM Tricine buffer, and penicillin/streptomycin (9, 10).

Construction of Expression Vectors

Full-length Aα and γ fibrinogen chain cDNAs were cloned into the PstI site of BR322 (17, 18) and were kind gifts from Dr. Dominic Chung, University of Washington, Seattle, WA. Both Aα and γ cDNA have internal PstI sites and both also have stop codons at the 5' end. Therefore, to obtain full length Aα and γ chain cDNAs, capable of being expressed, the following procedures were used to construct the expression vectors.

pBS12BI-Aα—The Aα cDNA was released from pBR322 by treatment with MspI. The resulting 3.2-kb fragment (200 ng) was then digested with nuclease Bal 31 for 4 min to remove 50 bp from both ends so that the stop codon at position -28 together with 22 bp of pBR322 sequence was removed. The resulting DNA fragment, which

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† The abbreviations used are: COS, monkey kidney fibroblasts; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RSV, Rous sarcoma virus; kb, kilobase(s); Neo, neomycin-kanamycin-resistant gene; bp, base pair(s).
contains the full length coding region for \( \text{Aa} \) fibrinogen chain, was purified and blunted with Klenow fragment followed by ligation with phosphorylated HindIII linker (10-mer, Boehringer Mannheim). This material was digested with HindIII/NcoI to create a HindIII site at the 5' end of the \( \text{Aa} \) cDNA and a NcoI site at the 3' end.

To prepare \( \text{pBC12BI-Aa} \) to receive the above fragment, \( \text{pBC12BI} \) was digested with \( \text{BglII/HindIII} \). The linear plasmid DNA was then ligated with phosphorylated BglII/Blunt adaptor (ds24/20-mer) from Boehringer Mannheim that contained a NcoI site at the other end. The plasmid DNA (100 ng) containing the adaptor was digested with NcoI and then ligated with \( \text{Aa} \) cDNA (described above). The resulting circular expression vector, having the \( \text{Aa} \) cDNA insert (Fig. 1A), was screened by digestion of a DNA mini-preparation with \( \text{BamHI/HindIII} \), which showed the presence of an insert of approximately 2 kb in the vector.

\( \text{pRSVNeo-Aa} \) cDNA was released from \( \text{pBC12BIAa} \) by digestion with \( \text{BamHI/HindIII} \). The released \( \text{Aa} \) cDNA (200 ng) was ligated to 100 ng of pRSV Neo (19) that had been cut with HindIII and dephosphorylated with calf intestinal phosphatase. The linear vector containing \( \text{Aa} \) cDNA, was filled-in with Klenow fragment and then self-ligated to form the circular expression vector (Fig. 1B). The correct orientation was determined by digesting the plasmid DNA with \( \text{BamHI/HindIII} \) and selecting the clones that yielded fragments of 4.3 and 3.4 kb. The 4.3-kb fragment is composed of 2.0 kb of \( \text{Aa} \) cDNA insert and 2.3 kb from the vector DNA.

To determine whether the cDNA inserts occurred in the correct orientation, these expression vectors, or with equal amounts of two of these expression vectors, or with equal amounts of all three expression vectors. In all cases 5 \( \mu \)g of DNA/ml of each vector was used. When only one, or a mixture of two vectors were used pRSVNeo DNA was added to increase the DNA concentration to 15 \( \mu \)g/ml. As a control, COS-1 cells were also transfected with pRSVNeo (15 \( \mu \)g/ml) that did not contain fibrinogen cDNA inserts. The transfected cells were selected by resistance to 0.4 mg/ml geneticin for 5 weeks as previously described (12). After 2 weeks, two or three colonies remained in each of the transfected cell lines. After another 3 weeks the colonies were treated with trypsin, transferred to fresh plates, and allowed to grow to confluency in 60-mm plates in the presence of 0.4 mg/ml geneticin.

**Incubation of Cells with \( L-[\text{35S}]\text{methionine} \)**

Before labeling with \( L-[\text{35S}]\text{methionine} \) the cells were kept for 24 h without geneticin in Iscove's medium supplemented with 10% fetal calf serum and 1% glucose. The 90% confluent cells were then labeled for 2 h at 37 °C in methionine-free Dulbecco's minimal essential medium (GIBCO) containing 200 \( \mu \)Ci of \( L-[\text{35S}]\text{methionine}, 0.1 \text{mg/ml} \text{heparin}, \) and 1% glucose (12).

**Immunoprecipitation of Nascent Fibrinogen Chains**

Radioactive fibrinogen chains were isolated by immunoprecipitation from cell lysates and from the cell medium. A rabbit polyclonal antibody that reacts with fibrinogen and its component chains was used (9, 10). Cell lysates were treated with iodoacetamide, detergents, and proteolytic inhibitors prior to immunoprecipitation. The chains were separated by SDS-PAGE under reduced and nonreduced conditions and detected by autoradiography. The nonreduced polypeptides were excised from the gels, reduced with mercaptoethanol, and re-electrophoresed on SDS-PAGE to identify the component chains. These procedures have previously been described (9-12).

Protein radioactivity was determined by cutting out the radioactive areas from the polyacrylamide gels and counting by liquid scintillation spectrometry (22). In some cases relative amounts of radioactivity were determined by scanning autoradiograms with a Shimadzu densitometer.

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Fig. 1. Diagram of expression vectors, containing \( \text{Aa} \) and \( \gamma \) fibrinogen chain cDNAs used to transfect COS-1 cells.
Clotting of Recombinant Fibrinogen

The incubation medium of COS-α,β,γ cells and of Hep G2 cells, incubated with L-[45S]methionine for 2 h at 37 °C, was collected. An aliquot (0.75 ml) was treated with 220 units/ml, final concentration, of Trasylol, and mixed with purified human plasma fibrinogen (1.4 mg/ml) and CaCl2 (0.024 M). Some samples also contained 5 mM iodoacetamide to inhibit Factor XIII that is usually found in most plasma fibrinogen preparations. This treatment blocks cross-linking that occurs with fibrinogen and other proteins. Clotting was initiated by the addition of 3 units/ml thrombin. The clot was allowed to form overnight in a sealed Centrifuge tube (Schleicher & Schuell) housing a 0.45-μm cellulose acetate filter in which the bottom of the upper stage was sealed. The next day the clot was percolated with 0.05 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4, until the radioactive background of the eluate had stabilized at its lowest level. The tube was then centrifuged to remove all liquid from the clot and the clot was stabilized at its lowest level. The tube was then incubated for 24 h at 37 °C and the clot was hydrolyzed in 0.2 M NaOH containing 40% urea. The hydrolyzed clot was neutralized with HCl and radioactivity determined. As a control, radioactive medium from COS cells transfected with an expression vector that did not contain fibrinogen chain cDNAs was treated in the same manner.

Quantitation of Secreted Fibrinogen

The amount of secreted fibrinogen present in the medium of cells incubated for 24 h at 37 °C was determined by an indirect competition enzyme-linked immunosorbent assay procedure using a monoclonal antibody (F94-7B3) that is specific for an epitope in the γ chain of human fibrinogen fragment D (25). In brief, the assay procedure was as follows. Polyvinyl microtiter plates (Costar) were coated with pure human fibrinogen. An appropriate dilution of antibody was mixed with an equal volume of either buffer, pure human fibrinogen (concentration range of standard curve: 0.25-4.0 μg/ml), or media (from COS-α,β,γ or Hep G2 cells). After mixing, each sample was added to the fibrinogen-coated enzyme-linked immunosorbent assay plate. Following incubation and subsequent wash cycles, an appropriate dilution of peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulin was added. Enzyme-linked IgG binding was detected using a H2O2- and o-dianisidine solution.

RESULTS

Expression of Single and Combinations of Fibrinogen Chain cDNAs by COS Cells—COS-1 cells were transfected with the expression vector pBC12BI containing either full length Aα and γ chain cDNA and then, 48 h later, the cells were incubated with L-[35S]methionine and the expression of radioactive fibrinogen chains determined. The cells expressed radioactive proteins which were immunoprecipitated with antibody to human fibrinogen. COS-cells transfected with pBC12BI-Aα produced a radioactive protein which comigrated with authentic Aα chains and COS cells transfected with pBC12BI-γ expressed γ chains (data not shown). Cells transiently transfected with pBC12BI-βγ have previously been shown to express fibrinogen βγ chains (16).

Knowing that COS cells are capable of expressing Aα, Bβ, and γ chains of fibrinogen, we then transfected COS cells with pRSVNeo-Aα, pRSVNeo-Bβ, and pRSVNeo-γ and selected stably transfected cell lines which were resistant to genetin. Geneticin-resistant COS cells transfected with any one of the expression vectors expressed proteins which reacted with rabbit antibody to human fibrinogen and were of similar size to appropriate authentic human plasma fibrinogen chains. Cells transfected with combinations of two vectors containing different fibrinogen chain cDNAs (Aα and Bβ, Aα and γ, and Bβ and γ) synthesized both chains and cells transfected with all three vectors expressed the three fibrinogen chains (Fig. 2).

Analyses, in nonreducing conditions, of the fibrinogen chains produced by cells transfected with combinations of two vectors showed that Aα and Bβ, Aα and γ, and Bβ and γ formed disulfide-linked complexes. The principal products and their molecular weights are Aα·Bβ complex (M, ~125,000), Aα·γ complex (M, 135,000), and Bβ·γ complex (M, 121,000) (Fig. 3). The size of the Aα·γ complex, as calculated from its electrophoretic mobility on SDS-PAGE, is larger than predicted but not large enough to suggest the presence of a third chain. In addition to these principal products, small amounts of larger size complexes were also noted, but no free chains were detected (Fig. 3A). The chain composition of the complexes was determined by reduction with mercaptoethanol and re-electrophoresis of the products. The complexes yielded a mixture of the expected two chains (Fig. 3B).

Synthesis of Fibrinogen by COS-α,β,γ Cells—COS-α,β,γ cells synthesized several fibrinogen-related proteins when analyzed under nonreducing conditions. The pattern noted is similar to that seen in Hep G2 cells (Fig. 4A). In COS-α,β,γ cells, after 2 h of metabolic labeling with L-[35S]methionine, 24.5% of the immunoprecipitable radioactivity was in fibrin-
oxygen, 22% in Aα-γ complex, 6% in free Aα chains, and 41% in free γ chains. A parallel experiment with Hep G2 cells showed 31% in fibrinogen, 20% in Aα-γ, 9% in free Aα, and 28% in free γ chains. Thus both stable transfected COS cells and Hep G2 cells develop surplus amounts of Aα and γ chains which reside intracellularly mainly as free γ chain and as an Aα-γ complex.

The major intracellular forms of fibrinogen in COS-αβ,γ cells were characterized, as had been done previously for Hep G2 cells, by excision of the radioactive bands from the polyacrylamide gel, reduction, and re-electrophoresis (10). Their chain compositions and estimated molecular weights allowed us to identify these as fibrinogen, Aα-γ complex, and free Aα and γ chains (Fig. 4B).

Secretion—COS cells which expressed single fibrinogen chains, and those which expressed two of the chains, in any combination, did not secrete these proteins into the medium (Fig. 5A). These single and duplex radioactive fibrinogen chains were only detected in the cell lysate (Figs. 2 and 3). However, COS-αβ,γ cells secreted the expressed proteins into the medium (Fig. 5A). When analyzed under nonreducing conditions the secreted fibrinogen chains were components of a high molecular weight disulfide-linked complex, with an apparent Mr of 340,000 which is similar to that of plasma fibrinogen. This Mr, 340,000 complex accounts for 98.4% of the immunoprecipitable protein radioactivity secreted. No free fibrinogen chains, nor intermediate products of assembly were detected in the medium (Fig. 5B). A small amount of protein radioactivity (less than 1%) was sometimes noted at about 130 kDa and this may be due to leakage from the cell or may be a degradative product of fibrinogen. A similar pattern was noted in the secretion of fibrinogen by Hep G2 cells (Fig. 5B). In the case of fibrinogen secreted by Hep G2 cells 90.4% of the immunoprecipitable radioactivity occurred as fibrinogen (Mr, 340,000) and a small amount, (~5%) was noted in a wide area, between 130 and 115 kDa (Fig. 5B).

Excision, reduction, and re-electrophoresis of the Mr 340,000 radioactive protein secreted by COS-αβ,γ cells showed that this large protein was composed of Aα, Bβ, and γ chains. A similar pattern was noted in the fibrinogen secreted by Hep G2 cells (Fig. 5).
a 48-kDa protein and on treatment with endoglycosidase H it migrated faster, as a 42-kDa protein (Fig. 6, lanes 3 and 4).

The Bα and γ chains of intracellular fibrinogen, synthesized by COS-α,β,γ cells were also both endoglycosidase H-sensitive (Fig. 6, lanes 5 and 6). Thus, the major intracellular forms of fibrinogen, Aα-γ complex, the free γ chain, and nascent fibrinogen, are all endoglycosidase H-sensitive, indicating that they accumulate or are retained in a pre-trans-Golgi membrane compartment. Similar results were obtained when nascent intracellular fibrinogen, synthesized by Hep G2 cells, was analyzed (data not shown).

By contrast, the glycoprotein chains of secreted fibrinogen, produced by either COS-α,β,γ (Fig. 6, lanes 7 and 8) or Hep G2 cells (not shown), are endoglycosidase H-insensitive. This suggests that recombinant fibrinogen follows the conventional secretory pathway with normal glycosylation.

Clotting of Recombinant Fibrinogen—To determine whether secreted recombinant fibrinogen is capable of clotting, the incubation medium of control (nontransfected) and COS-α,β,γ cells, incubated for 24 h with L-[35S]methionine, was mixed with human plasma fibrinogen and induced to clot by the addition of thrombin. The clotting ability of recombinant fibrinogen was compared to that of fibrinogen secreted by Hep G2 cells. Clots formed in the presence of radiolabeled media from COS cells that were not transfected with the fibrinogen chain cDNAs had only background amounts of radioactivity associated with the clot matrix (2–3% of total trichloroacetic acid-precipitable radioactivity from the media). By contrast, clots formed in the presence of radiolabeled media from COS-α,β,γ cells, or from Hep G2 cells, had 50 to 45 times background levels of radioactivity associated with the clot. Clotting in the absence of Factor XIII cross-linking (i.e. in the presence of iodoacetamide) also produced highly radiolabeled clots, 18 to 24 times the background level. This indicates that the radiolabeled secreted fibrinogen became associated with the clot matrix through a thrombin-dependent polymerization mechanism.

Amount of Recombinant Fibrinogen Secreted—COS-α,β,γ cells secreted comparable amounts of fibrinogen as compared to Hep G2 cells. In two experiments COS-α,β,γ cells (2 × 10⁶ cells) secreted an average of 2.08 μg of fibrinogen in 24 h and the same number of Hep G2 cells secreted 1.94 μg of fibrinogen.

DISCUSSION

Fibrinogen is a multichain protein with a well ordered structure. It is sensitive to thrombin and acts in the final stages of blood clotting. Fibrinogen assembly which involves the arrangement of three different polypeptides into a symmetrical dimer probably occurs on structures within the endoplasmic reticulum which mediate proper alignment of the chains and also specific disulfide interactions. As such, a group of proteins known to be present in the lumen of the endoplasmic reticulum which probably include the immunoglobulin binding protein and protein disulfide isomerase are likely to be involved in a concerted effort to assemble the various chains into a functional molecule. (For reviews see Refs. 27, 28). Previously, the individual chains of fibrinogen have been expressed in surrogate cells, either E. coli (29–31) or in COS cells (16). However, expression, assembly, and secretion of fully formed, functional recombinant fibrinogen has not been reported. We show that COS cells, transfected with single fibrinogen chain cDNAs or with any combination of two fibrinogen chain cDNAs, express the appropriate fibrinogen chains but cannot secrete them. In contrast, COS cells containing all three fibrinogen chain cDNAs express, assemble, and secrete the chains in a form which is capable of forming a thrombin-induced clot. This indicates that factors needed for proper assembly of fibrinogen chains are not restricted to two tissues, hepatocytes and megakaryocytes, which normally express fibrinogen (32, 33). This further demonstrates that for fibrinogen chains to be properly transported and secreted they must exist as part of fully formed dimeric fibrinogen. This suggests that intact fibrinogen contains a signal which allows intracellular transport and secretion to occur and that individual chains are recognized as products not to be secreted.

In vivo free fibrinogen chains have not been detected in the circulation. In dogs, injected with radioactive amino acids, nearly all of the secreted fibrinogen chain radioactivity is accounted for in fibrinogen (34) and studies with cells in culture have indicated that fully formed fibrinogen is the main, if not the only form, of secreted fibrinogen chains (35, 36). This occurs in spite of the fact that in hepatocytes of several species studied, there is a surplus of two of the component chains of fibrinogen (9, 10, 35–37). In dogs (34) and rabbits (38) surplus chains have not been detected intracellularly, but different specific radioactivities of the component chains of secreted fibrinogen indicate that pools of Aα and γ chains may occur. Thus, hepatocytes have a mechanism for distinguishing the surplus forms of fibrinogen chains from fully formed fibrinogen. A similar mechanism occurs in transfected COS cells. The stable transfected COS cell lines only secrete fully formed fibrinogen. Under nonreducing conditions 99.4% of the secreted immunoprecipitable protein is fibrinogen. There is less than 1% protein radioactivity in lower molecular weight proteins. As evidenced by sensitivity to endoglycosidase H treatment, the nonsecreted fibrinogen chains, and also nascent fibrinogen which is not yet fully processed, are retained in a pre-Golgi compartment. This is similar to the assembly and degradation of other heterologous proteins. In both the human asialoglycoprotein receptor (39) and the T-cell receptor proteins (40, 41) surplus chains are produced and some of these excess chains are degraded in a nonlysosomal pre-Golgi compartment.

Previous pulse-chase experiments, which carefully measured kinetic precursor-product relationships in Hep G2 cells, showed that surplus Aα and γ fibrinogen chains participate in fibrinogen synthesis and assembly and that unused chains are retained and degraded intracellularly (9, 10). In COS-α,β,γ cells incubated for 2 h with L-[35S]methionine, which is near steady-state conditions, most of the radioactivity in fibrinogen chains occurs in three forms; as fully assembled fibrinogen whose carbohydrates have not been completely processed, as an Aα-γ complex and as free γ chains (Fig. 4). In addition, some free Aα chains, and other intermediate forms, account for a small percentage of intracellular fibrinogen chains. This pattern is similar to that noted in Hep G2 cells and suggests that COS-α,β,γ cells assemble chains in a similar manner to Hep G2 cells. Kinetic pulse-chase experiments have not yet been performed with transfected COS cells and it is not clear whether all the intracellular precursor fibrinogen forms detected in Hep G2 cells are also present in transfected COS cells; or whether all of the intermediate forms present in COS cells participate in fibrinogen assembly. However, it is apparent that, in hepatocytes of all species studied, surplus γ chains are generated in COS cells during fibrinogen assembly.

The mechanism by which surplus γ chains are generated is not understood. In Hep G2 cells, the initial rates of synthesis of the three chains are unequal with that of Bβ being less than that of Aα and γ (10). However, unequal degradative
rates have not been ruled out. In transfected COS cells the expression of the three fibrinogen chains is driven by the same viral promoter present in the expression vector pRSVNeo and thus regulation is unlikely to occur at the nuclear level; although we cannot rule out that different mRNAs are exported from the nucleus at different rates or that they have different stabilities. More likely the generation of surplus γ chains in transfected COS cells is a consequence of the chain assembly process and is probably a posttranslational event.

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