Recombinant Human Fibrinogen and Sulfation of the γ′ Chain†

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Received May 21, 1991; Revised Manuscript Received July 24, 1991

ABSTRACT: Human fibrinogen and the homodimeric γ′-chain-containing variant have been expressed in BHK cells using cDNAs coding for the α, β, and γ (or γ′) chains. The fibrinogens were secreted at levels greater than 4 μg (mg of total cell protein)$^{-1}$ day^{-1} and were biologically active in clotting assays. Recombinant fibrinogen containing the γ′ chain incorporated $^{35}SO_4$ into its chains during biosynthesis, while no incorporation occurred in the protein containing the γ chain. The identity of the sulfated γ′ chain was verified by its ability to form dimers during clotting. In addition, carboxypeptidase Y digestion of the recombinant fibrinogen containing the γ′ chain released 96% of the ^{35}S label from the sulfated chain, and the radioactive material was identified as tyrosine *O*-sulfate. These results clarify previous findings of the sulfation of tyrosine in human fibrinogen.

Human fibrinogen is a soluble plasma protein that is converted to insoluble fibrin in the presence of thrombin. Fibrinogen (M_r 340 000) is composed of two sets of three polypeptides, the α (M_r 66 000), β (M_r 52 000), and γ (M_r 46 500) chains (McKee et al., 1966). The six chains, $(\alpha\beta\gamma)_2$, are extensively linked by disulfide bonds to form a complex trinodular structure (Hall & Slayter, 1959). During the coagulation cascade, the conversion of fibrinogen to a fibrin monomer occurs by the cleavage of amino-terminal fibrinopeptides from the α and β chains (Bailey et al., 1951). This exposes polymerization sites which allow the fibrin monomers to interact and form the clot matrix (Laudano & Doolittle, 1978). The matrix is stabilized by the formation of γ-glutamyl-ε-lysine cross-links involving the α and γ chains. This cross-linking reaction is catalyzed by factor XIIIa in the presence of calcium ions (Lorand et al., 1980; McKee et al., 1970) and results in a highly stable insoluble clot.

Fibrinogen is a multifunctional protein with many discrete domains. For example, fibrinogen (or fibrin) has binding sites for plasminogen (Lucas et al., 1983), tissue plasminogen activator (van Zonneveld et al., 1986), thrombin (Liu et al., 1979), and other plasma components. Fibrinogen also participates in platelet aggregation by binding to specific receptors on activated platelets (Marguerie et al., 1979; Hawiger et al., 1980). Accordingly, fibrinogen plays a central role in hemostasis and thrombosis.

The amino acid sequence of each of the three chains of human fibrinogen has been determined by amino acid sequence analysis (Blombäck et al., 1976; Doolittle et al., 1979; Henschen et al., 1980; Watt et al., 1979). In addition, the sequences of the cDNAs coding for the three chains (Chung et al., 1983a,b; Kant et al., 1983; Rixon et al., 1983) and their genes (Chung et al., 1990) have also been established. The three genes are clustered on chromosome 4 at position 4q23–32 (Henry et al., 1984) and occur in the order of α, γ, and β. The gene for the β chain is in the reverse orientation relative to the α and γ genes (Kant et al., 1985). The α, β, and γ genes for human fibrinogen span approximately 45 kb and contain four, seven, and nine introns, respectively (Chung et al., 1990; Kant et al., 1985).

A variant, nonallelic form of the γ chain which is found in about 10% of human plasma fibrinogen molecules (Francis et al., 1980; Wolfenstein-Todel & Mosesson, 1980) arises from the use of an alternative polyadenylation site within the ninth intron (Chung & Davie, 1984; Fornace et al., 1984) and is referred to as γ′ (Wolfenstein-Todel & Mosesson, 1980), γB (Francis et al., 1980), or $\gamma^{57.5}$ (Peerschke et al., 1986). In the γ′ chain, the carboxyl-terminal 4 amino acids have been replaced by a 20 amino acid segment (Wolfenstein-Todel & Mosesson, 1981). The function of the γ′ chain is not known. Progress on its functional characterization has been hampered by the fact that γ′-containing fibrinogen has only been isolated as a heterodimer with the composition $(\alpha\beta\gamma)(\alpha\beta\gamma')$. Homodimeric $(\alpha\beta\gamma')_2$ fibrinogen has not been isolated from plasma, thus providing a major impetus for the expression system presented here.

The three fibrinogen chains have been expressed individually in *Escherichia coli* (Lord, 1985; Bolyard & Lord, 1988, 1989), but functional fibrinogen has not been synthesized in a prokaryotic expression system. The first biologically active recombinant human fibrinogen was synthesized from cDNA clones in a mammalian cell expression system using baby hamster kidney (BHK)[1] cells (Farrell et al., 1989). Subsequently, a COS-1 cell expression system for the stable expression of fibrinogen from cDNA clones has also been developed (Roy et al., 1991); however, biological activity of the fibrinogen from this system was not shown. Another COS-1 expression system which produced biologically active, clottable fibrinogen has also been described by Hartwig and Danishefsky (1991) employing a transient expression system to identify potential intermediates in the assembly process.

Human fibrinogen has long been known to be sulfated on tyrosine residues (Jevons, 1963). Using the HepG2 hepatocellular carcinoma cell line (Knowles et al., 1980) which secretes human fibrinogen, Liu et al. (1985) identified the β chain of fibrinogen as the chain containing the sulfated tyrosine, based on the mobility of the β chain on SDS gels. The

† This work was supported by National Institutes of Health Grant HL 16919.

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[1] Abbreviations: BHK, baby hamster kidney; ELISA, enzyme-linked immunosorbent assay; rFbg, recombinant fibrinogen (containing γ chains); rFbgγ′, recombinant fibrinogen (containing γ′ chains).

0006-2960/91/0430-9414$02.50/0

site of sulfation, however, was different from that seen in bovine fibrinogen, which is sulfated on fibrinopeptide B (Bettelheim, 1954). Human fibrinogen lacks the corresponding tyrosine residue in its β-chain fibrinopeptide (Henschen et al., 1980; Watt et al., 1979). In addition, later studies in rat hepatocytes showed that rat fibrinogen was sulfated on tyrosine residues in the carboxyl end of the γ′ chain (Hirose et al., 1988). In this paper, we present evidence that human fibrinogen is sulfated on the γ′ chain, rather than the β chain as previously reported (Liu et al., 1985).

Experimental Procedures

Construction of Expression Vectors. pAG-1 encoding the α and γ cDNAs was constructed from the original cDNA clones in pBR322 (Rixon et al., 1983; Chung et al., 1983). The α cDNA was subcloned as an *Ase*I/*Pvu*I fragment, filled in with T7 DNA polymerase, and cloned as a blunt-end fragment into the *Hin*cII site of M13mp18 to create pAM-1. A *Bam*HI site was constructed three nucleotides upstream from the initiator methionine with the oligonucleotide 5′-CATGCCTGCAGGTCGGATCCAAGATGTTTTCCA-TGAG-3′ using an in vitro mutagenesis system (Amersham) to create pAM-2. In addition, a 793 base pair *Bgl*II/*Mlu*I fragment from the α genomic clone pBS4 (Chung et al., 1990) was used to correct a mistake in the original α cDNA in pHIα3 that contained a deletion of the codon TCT for Ser-417. The γ cDNA was subcloned by cleavage at the 5′ *Sst*I site and the 3′ *Hin*dIII site and inserted into the *Sst*I and *Hin*dIII sites in pIC19R (Marsh et al., 1984) to create pGI-1. A *Bgl*II/*Bam*HI fragment was subcloned from pGI-1 into the *Bam*HI site of Zem86 (Mulvihill et al., 1988) to create pGI-2. This placed the γ cDNA under the control of the SV-40 early promoter/enhancer element (Subramani et al., 1981). The human growth hormone transcriptional terminator was added at the 3′ end, since the *Hin*dIII cleavage removed the endogenous γ terminator. The terminator was identical with that from MThGH111 (Palmiter et al., 1983). The entire γ transcription unit including promoter and terminator was subcloned as an *Eco*RI fragment into pMT-1, creating pMG-1. This placed a metallothionein promoter (Palmiter et al., 1983) in the opposite transcriptional orientation to the SV-40 promoter. The metallothionein promoter was originally derived from MThGH111, as cloned in Zem93 (Mulvihill et al., 1988). The final step in the construction was the insertion of the modified α cDNA as a *Bam*HI fragment from pAM-2 into the *Bam*HI site in pMG-1 to create pAG-1. Plasmid pAG-γ′, which encodes the α and γ′ chains, was constructed in the same sequence shown for pAG-1, except that the 3′ end of the γ DNA in pGI-2 was replaced with the 758 bp *Pst*I fragment encoding the γ′-carboxyl terminus. In both pAG-1 and pAG-γ′, the α cDNA is transcribed from the metallothionein promoter, and the γ (or γ′) cDNA is transcribed in the opposite direction from the SV-40 promoter.

The β cDNA was cloned into the *Pst*I site of M13mp18 to create pBM-1. A new *Pst*I site was constructed three nucleotides upstream from the initiator methionine with the oligonucleotide 5′-CTTGCATGCCTGCAGACCATGA-AACATCTATTA-3′ to create pBM-2. The SV-40 promoter/enhancer element and the dihydrofolate reductase cDNA originally present in pSV2 DHFR (Subramani et al., 1981) were subcloned as an *Eco*RI fragment from Zem176 (Mulvihill et al., 1988) into Zem93 to create pMD-1, which has a metallothionein promoter in the opposite transcriptional orientation to the SV-40 promoter. The modified β cDNA from pBM-2 was subcloned as a *Pst*I fragment into the *Pst*I site in pMD-1 to create pBD-1. Transcription of the β cDNA from the metallothionein promoter was in the opposite direction from transcription of the dihydrofolate reductase cDNA from the SV-40 promoter.

The construction of expression vectors pAG-1, pAG-γ′, and pBD-1 was carried out by established techniques (Sambrook et al., 1989). The sequences of the modified cDNAs were confirmed by using a dideoxy chain termination system (United States Biochemical Corp).

Cell Culture. HepG2 human liver cells (Knowles et al., 1980) were grown in minimum essential medium/10% fetal bovine serum/1 mM sodium pyruvate/0.1 mM nonessential amino acids/100 μg/mL neomycin/50 μg/mL penicillin/50 μg/mL streptomycin (Gibco) in a 5% CO_2 atmosphere at 37 °C. A thymidine kinase deficient baby hamster kidney cell line, BHK 570 (ATCC CRL 10314), containing the expression vector pPAB-5 (Busby et al., 1991) for α_2-antiplasmin (a generous gift from Dr. Don Foster; ZymoGenetics, Inc., Seattle, WA) was used as the host cell for the transfections and was grown under the same conditions in Dulbecco's modified Eagle medium/5% fetal bovine serum/100 μg/mL neomycin/50 μg/mL penicillin/50 μg/mL streptomycin (Gibco). For transfections, BHK cells were plated at 1:25 split ratios in 150-mm plates (Falcon) overnight and transfected with 25 μg of calcium phosphate precipitated pAG-1 (or pAG-γ′) and pBD-1 (12.5 μg of each) for 4 h in 10 mL of medium. After a 1-min shock in 15% glycerol/Tris-buffered saline, cells were grown for 24 h in normal medium. The cells were then grown in selective medium with 20 μM methotrexate for 7–10 days and screened by using an immunofilter assay (McCracken & Brown, 1984). The highest producing clones isolated were designated BHK-Fbg and BHK-Fbgγ′, which secreted fibrinogen (rFbg) and γ′-containing homodimeric fibrinogen (rFbgγ′), respectively.

Immunoprecipitation and Clotting Assays. Confluent BHK-570, BHK-Fbg, HepG2, and BHK-Fbgγ′ cells in 24-well plates (Corning) were washed twice with 2 mL of 120 mM NaCl/2.7 mM KCl/10 mM sodium phosphate, pH 7.4 (phosphate-buffered saline), and metabolically labeled for 24 h in 0.5 mL of Dulbecco's modified Eagle medium containing 20 mM Hepes (pH 7.4)/3.7 g/L sodium bicarbonate/100 μg/mL neomycin/50 μg/mL penicillin/50 μg/mL streptomycin. For [^{35}S]cysteine labeling, 100 μCi/mL [^{35}S]cysteine (>600 Ci/mmol, Amersham) was added to cysteine-free medium (JRH Biosciences). For $^{35}SO_4$ labeling, 100 μCi/mL $^{35}SO_4$ (25–40 Ci/mg, Amersham) was added to sulfate-free medium (JRH Biosciences). Control cells without label were also used in ELISA assays to be described below in order to quantitate expression levels. Total cell protein in the cell monolayers was determined by using the BCA assay (Pierce) on cells extracted in RIPA buffer (Sambrook, 1989), using bovine serum albumin as a standard.

For immunoprecipitations, the medium described above containing 50 μg/mL benzamidine (Sigma)/1 μM leupeptin (Boehringer Mannheim)/50 μg/mL soybean trypsin inhibitor (Sigma) was used for labeling. After the 24-h incubation, the medium was removed, and protease inhibitors were added at the following concentrations: 5 mM 6-amino-*n*-hexanoic acid (Sigma)/5 mM EDTA (Sigma)/0.1 mM *n*-ethylmaleimide (Sigma)/1 μM pepstatin A (Boehringer Mannheim)/0.2 mM phenylmethanesulfonyl fluoride (Sigma). The cells were washed twice with 2 mL of phosphate-buffered saline and solubilized in 0.5 mL of RIPA buffer containing the above protease inhibitors. All subsequent incubations were done at 4 °C with rocking. The labeled material was preadsorbed with 5 μL of normal rabbit serum for 1 h and precipitated with 2

mg of protein A–Sepharose (Sigma) for 1 h. The protein A–Sepharose was pelleted by centrifugation in a microfuge for 30 s at 4 °C. A total of 2.5 μL of a rabbit anti-human fibrinogen antiserum (Behring) was added to the remaining supernatant and incubated for 1 h. Two milligrams of protein A–Sepharose was added for 1 h and washed with 1 mL of RIPA, 1 mL of 0.5 M NaCl/20 mM Tris (pH 7.4)/1% NP-40/1 mM EDTA, and 1 mL of 0.15 M NaCl/20 mM Tris (pH 7.4)/1 mM EDTA (all wash buffers contained the above protease inhibitors). Sample buffer (100 μL) with or without 5% 2-mercaptoethanol was added to the pellet and boiled 5 min before being loaded on gels (Laemmli, 1970).

For clotting assays, cells were labeled in the absence of protease inhibitors. After 24 h, the medium was removed, and 25 μL of human plasma (George King Biomedical) was added and allowed to clot for 3 h at room temperature. The clots were centrifuged 10 min at 4 °C in a microfuge, and the pellets were washed and solubilized with the same buffers used for the immunoprecipitations.

^{14}C-Labeled molecular weight markers were obtained from Bethesda Research Laboratories. The samples were run on 10% gels according to Laemmli (1970), impregnated with Amplify (Amersham) according to the manufacturer's directions, dried, and exposed to XAR-5 film (Kodak) with intensifying screens (Cronex) at −70 °C.

Assay for Fibrinogen. An ELISA for fibrinogen was developed by using the procedure of Flaherty et al. (1990). Briefly, an affinity-purified IgG fraction from a rabbit polyclonal antiserum to human fibrinogen (Accurate Chemical & Scientific Corp.) was biotinylated using biotin–amidocaproate *n*-hydroxysuccinimide ester (Sigma) and used for the detection of antibody-bound immobilized fibrinogen in ELISA plate wells (Corning). Streptavidin–alkaline phosphatase (BRL) was used to detect the biotinylated antibody. The phosphatase substrate used was *p*-nitrophenyl phosphate (Sigma). The limit of detection of the assay was below 1 ng/mL fibrinogen. Normal plasma fibrinogen isolated by glycine precipitation (Kazal et al., 1963) was used as the standard.

Tyrosine O-Sulfate Analysis. ^{35}S-Labeled rFbgγ′ was isolated by immunoprecipitation of $^{35}SO_4$-labeled BHK-Fbgγ′ cells as described above, except that 100-mm plates of cells were labeled with 5 mL of medium containing 200 μCi/mL $^{35}SO_4$. The immunoprecipitate from 1 mL of medium, which included the protein A–Sepharose, antibody, and labeled rFbgγ′, was digested for 24 h at 37 °C in 100 μL of 50 mM sodium acetate, pH 5.5, containing 0.5 μg/mL carboxypeptidase Y (Calbiochem; 143.6 units/mg). The reaction mixture was centrifuged in a microfuge for 30 s, and 10 μL of the supernatant was precipitated with 90 μL of acetone for 30 min at 4 °C. The precipitate was removed by centrifugation for 10 min in a microfuge, and the supernatant was evaporated until it was dry.

For amino acid analysis, the dried acetone supernatant (approximately 2600 cpm) was derivatized with phenyl isothiocyanate and chromatographed on a WISP C18 system (Waters) as previously described (Bidlingmeyer, 1984). Fractions (0.5 mL) were collected every 0.5 min and mixed with 5 mL of Ecolume (ICN) for scintillation counting. Tyr *O*-sulfate standard (200 pmol), kindly provided by Dr. Ming-Cheh Liu (University of Oklahoma, Norman, OK), was derivatized and chromatographed in the same manner, and the effluent was monitored at 254 nm.

Results

Secretion of Recombinant Fibrinogens from BHK Cells. The baby hamster kidney cell line BHK 570, which is deficient in thymidine kinase, was chosen as the host cell line for transfection because of its ability to express many proteins of the coagulation and fibrinolytic pathways, and its ability to allow amplification of expression vectors containing the dihydrofolate reductase selectable marker. A derivative of this cell line which expresses α_2-antiplasmin was used in order to minimize proteolysis of the expressed fibrinogen secreted into the medium. Cotransfection of these BHK cells with pAG-1 and pBD-1 (Figures 1 and 2) and selection in 20 μM methotrexate resulted in several colonies secreting rFbg. The parental BHK cell line did not produce detectable fibrinogen, using an assay capable of detecting less than 1 ng/mL. A stable cell line (BHK-Fbg) produced rFbg at levels of 1.1 μg mL^{-1} day^{-1} at confluence in 24-well plates. To produce homodimeric γ′-containing fibrinogen (rFbgγ′), BHK cells were transfected with pAG-γ′ and pBD-1, resulting in cell line BHK-Fbgγ′. Similar secretion levels of 0.83 μg mL^{-1} day^{-1} were achieved with BHK-Fbgγ′, which produced rFbgγ′. Each recombinant cell line produced slightly less fibrinogen at confluence than the HepG2 cell line (1.3 μg mL^{-1} day^{-1}). When the data were normalized to the total amount of cellular protein in the wells, however, the recombinant cell lines produced more fibrinogen per unit of total cellular protein. HepG2 cells produced 3.0 μg (mg of protein)$^{-1}$ day^{-1}, while BHK-Fbg and BHK-Fbgγ′ produced 5.0 and 4.0 μg (mg of protein)$^{-1}$ day^{-1}, respectively.

rFbg comigrated with normal human fibrinogen from HepG2 cells on unreduced SDS–polyacrylamide gel electrophoresis (Figure 3, lanes 2 and 3). Upon reduction, the recombinant α, β, and γ chains also comigrated with their normal counterparts (lanes 6 and 7). Similarly, rFbgγ′ migrated as a high molecular weight complex under nonreducing conditions (lane 4). Upon reduction, the α and β chains comigrated with the HepG2 chains, while the γ′ chain migrated at a slightly higher position than the γ chain, consistent with the greater molecular weight of the γ′ chain (lanes 7 and 8). These results indicate that the recombinant fibrinogens had the correct $(\alpha\beta\gamma)_2$ or $(\alpha\beta\gamma')_2$ composition.

Biological Activity of the Recombinant Fibrinogens. The recombinant fibrinogens were assayed for functional activities which are essential physiological features of normal fibrinogen. These include the ability of fibrinogen to be incorporated into a fibrin clot and the ability of fibrin monomers to be cross-linked by factor XIIIa. Incorporation into a fibrin clot and subsequent cross-linking require at minimum (1) cleavage of fibrinopeptide A by thrombin, exposing polymerization sites in the resulting fibrin monomers, and (2) proper alignment in the fibrin matrix, such that the α and γ (or γ′) chains in adjacent fibrin monomers are correctly oriented for cross-linking by factor XIIIa.

In order to test the clottability of the recombinant fibrinogens, it was necessary to use an assay which could detect relatively low amounts of recombinant fibrinogens produced by the BHK cells. Standard clotting assays which rely on 2–4 mg/mL fibrinogen were unsuitable. Metabolically labeled recombinant fibrinogens were therefore used for the clotting assays. Figure 3 shows that recombinant fibrinogens labeled with [^{35}S]cysteine were incorporated into a fibrin clot and were readily cross-linked. In lanes 9–12, the labeled medium was clotted with normal human plasma prior to electrophoresis. The washed, solubilized clots from BHK, BHK-Fbg, HepG2, and BHK-Fbgγ′ media are shown in lanes 9, 10, 11, and 12, respectively. In both BHK-Fbg and HepG2 fibrin clots, the α- and γ-chain bands diminished in intensity, consistent with their conversion by factor XIIIa to multimers and cross-linked

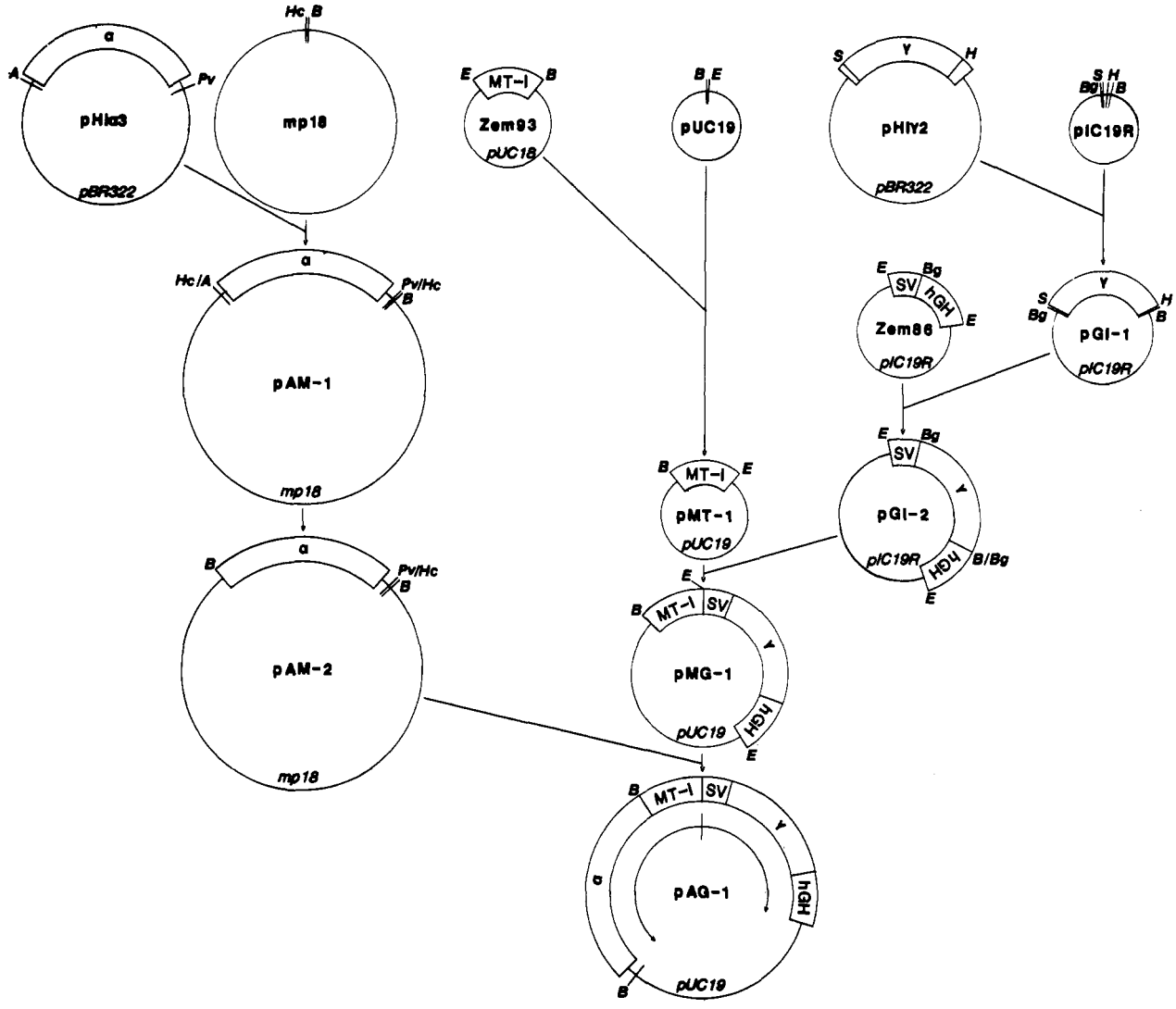

FIGURE 1: Construction of pAG-1. The 5'-untranslated regions of the original α and γ cDNAs in pHIα3 and pHIγ2 were removed after being cloned into M13mp18 and pIC19R, respectively, to create pAM-2 and pGI-1. The α cDNA was placed under the control of a modified metallothionein promoter, while the γ cDNA was placed under the control of the SV-40 early promoter/enhancer. The direction of transcription is shown by the curved arrows. *A* = *Ase*I, *B* = *Bam*HI, *Bg* = *Bgl*II, *E* = *Eco*RI, *H* = *Hin*dIII, *Hc* = *Hin*cII, *Pv* = *Pvu*I, *S* = *Sst*I.

dimers, respectively (lanes 10 and 11). The β-chain bands shifted to a lower position on the gel, probably due to proteolytic degradation, as seen by other investigators (Lucas et al., 1983). Concomitant with the disappearance of the γ bands, new bands appeared at the position of cross-linked γ–γ dimers.

Similarly, rFbgγ′ was also incorporated into a fibrin clot. Lane 12 shows that after clotting, the β band comigrated with those from both BHK-Fbg and HepG2 fibrinogen. The α band and γ′ band decreased in intensity, while a new band appeared at a position slightly above the γ–γ dimer. Since the unlabeled human plasma used to form the clot contributed much more fibrinogen in this assay than the BHK-Fbgγ′ cells (~75 μg vs ~0.5 μg), this new band is most likely a γ′–γ heterodimer. These results indicate that both the recombinant and HepG2 fibrinogens were incorporated into fibrin clots in the correct orientation, such that the α and γ (or γ′) chains acted as substrates for factor XIIIa. Furthermore, in preliminary experiments, it was shown that rFbg bound to platelets in a dose-dependent manner which paralleled the binding of plasma fibrinogen (unpublished results). Therefore, the recombinant fibrinogens appear to be biologically active using several important criteria, including incorporation into fibrin clots and cross-linking by factor XIIIa.

Sulfation of Fibrinogen. The recombinant fibrinogens were examined for sulfation in order to determine if any differences in this posttranslational modification existed between rFbg and rFbgγ′. BHK-Fbg and BHK-Fbgγ′ cells were labeled with $^{35}SO_4$ to visualize the sulfated chains, and parallel cell cultures were labeled with [^{35}S]Cys to unambiguously identify the three chains of fibrinogen. The labeled medium was then either immunoprecipitated or clotted with normal human plasma. Figure 4 shows that only the γ′ chain incorporated detectable amounts of $^{35}SO_4$. Lanes 1 and 2 show rFbg labeled with [^{35}S]Cys and $^{35}SO_4$, respectively, and immunoprecipitated. No $^{35}SO_4$-labeled bands are seen in lane 2. In contrast, rFbgγ′ labeled with [^{35}S]Cys and $^{35}SO_4$ (lanes 3 and 4, respectively) showed labeling of a band with $^{35}SO_4$ which comigrated with the γ′ band. These data show that only the γ′ chain was sulfated to an appreciable extent.

To ensure that the $^{35}SO_4$-labeled band was indeed the γ′ band and not a proteolyzed β or α band, the labeled media were clotted, and the solubilized clot was run on a gel. As shown earlier in Figure 3, the clotted fibrinogen had a characteristic binding pattern in which the α bands decreased sharply in intensity, the β band shifted to a slightly lower position, and the γ (or γ′) band shifted to the dimer position. Figure 4, lanes 5 and 6, shows [^{35}S]Cys- and $^{35}SO_4$-labeled

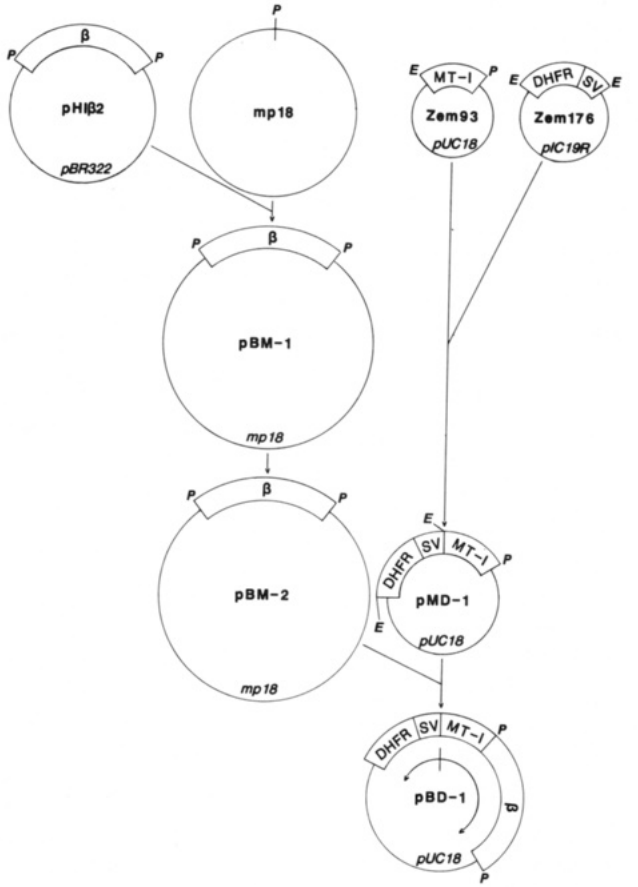

FIGURE 2: Construction of pBD-1. The 5′-untranslated region of the β cDNA in pHIβ2 was removed after being cloned into M13mp18 to create pBM-2. The β cDNA was placed under the control of a modified metallothionein promoter, while the dihydrofolate reductase selectable marker cDNA was under the control of the SV-40 early promoter/enhancer. The direction of transcription is shown by the curved arrows. *E* = *Eco*RI, *P* = *Pst*I.

rFbg, respectively, after clotting. The β band and the γ–γ dimer are apparent in the [^{35}S]Cys-labeled rFbg (lane 5), but not in the $^{35}SO_4$-labeled rFbg (lane 6). However, rFbgγ′ shows the γ–γ′ dimer band after labeling with both [^{35}S]Cys (lane 7) and $^{35}SO_4$ (lane 8), indicating that the γ′ chain was readily sulfated. Significantly, no $^{35}SO_4$-labeled band was detected at the position of the β or α bands. These results confirm the sulfation of the γ′ chain.

Tyrosine O-Sulfate Analysis. The $^{35}SO_4$-labeled rFbgγ′ was analyzed to determine whether the label was incorporated into Tyr residues. Carboxypeptidase Y treatment of the immunoprecipitated rFbgγ′ caused the release of 45% of the label after 30 min and 96% of the label after a 24-h digestion. The released labeled material was derivatized with phenyl isothiocyanate and chromatographed on a C18 column for amino acid analysis. A major peak of radioactivity which corresponded to 81% of the input ^{35}S label eluted 11.5 min after injection (Figure 5, upper panel), in addition to a minor breakthrough peak at 3.5 min and an unidentified minor peak at 15.5 min. In comparison, a derivatized Tyr *O*-sulfate standard eluted at 11.8 min after injection (Figure 5, lower panel), with a byproduct peak at 19.8 min. The elution times of the major ^{35}S peak and the Tyr *O*-sulfate standard indicate that the majority of the sulfation in the γ′ chain occurs on Tyr.

Discussion

Fibrinogen is the central molecule in the blood coagulation cascade and forms the structural basis of the fibrin clot. As such, it is essential for normal hemostasis in vivo. Although

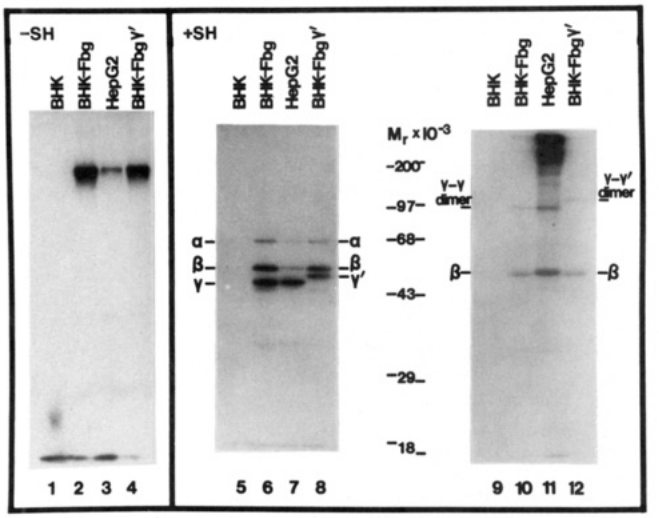

FIGURE 3: ^{35}S-Labeled fibrinogens. [^{35}S]Cysteine-labeled fibrinogens from BHK, BHK-Fbg, HepG2, and BHK-Fbgγ′ cells were immunoprecipitated or clotted and run on gels. Lanes 1–4 show normal and recombinant fibrinogens immunoprecipitated and run on 5% nonreduced gels; (1) BHK cells; (2) BHK-Fbg cells; (3) HepG2 cells; (4) BHK-Fbgγ′ cells. Lanes 5–8 show normal and recombinant fibrinogens immunoprecipitated and run on 10% reduced gels: (5) BHK cells; (6) BHK-Fbg cells; (7) HepG2 cells; (8) BHK-Fbgγ′ cells. Lanes 9–12 show normal and recombinant fibrinogens clotted with human plasma and run on 10% reduced gels: (9) BHK cells; (10) BHK-Fbg cells; (11) HepG2 cells; (12) BHK-Fbgγ′ cells.

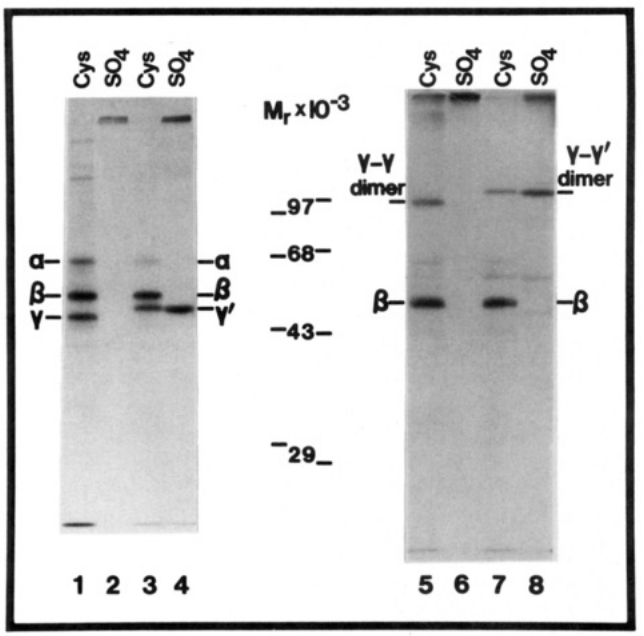

FIGURE 4: [^{35}S]Cysteine- and $^{35}SO_4$-labeled fibrinogens. [^{35}S]Cysteine- and $^{35}SO_4$-labeled fibrinogens from BHK-Fbg and BHK-Fbgγ′ cells were immunoprecipitated or clotted and run on gels. Lanes 1–4 show recombinant fibrinogens immunoprecipitated and run on 10% reduced gels: (1) [^{35}S]Cys-labeled BHK-Fbg cells; (2) $^{35}SO_4$-labeled BHK-Fbg cells; (3) [^{35}S]Cys-labeled BHK-Fbgγ′ cells; (4) $^{35}SO_4$-labeled BHK-Fbgγ′ cells. Lanes 5–8 show recombinant fibrinogens clotted and run on 10% reduced gel: (5) [^{35}S]Cys-labeled BHK-Fbg cells; (6) $^{35}SO_4$-labeled BHK-Fbg cells; (7) [^{35}S]Cys-labeled BHK-Fbgγ′ cells; (8) $^{35}SO_4$-labeled BHK-Fbgγ′ cells.

a great deal is known about its structure and function, the unambiguous assignment of many biological functions to specific domains has yet to be made. The expression system presented here provides the capability to study structure/function relationships directly by site-specific mutagenesis.

Secreted rFbg was indistinguishable from normal fibrinogen by several criteria. Structurally, unreduced rFbg comigrated

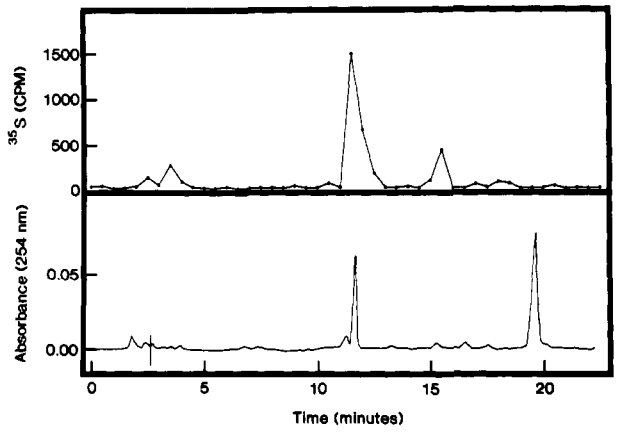

FIGURE 5: Tyrosine *O*-sulfate analysis. In the upper panel, $^{35}SO_4$-labeled rFbgγ' was digested with carboxypeptidase Y, derivatized with phenyl isothiocyanate, and chromatographed on a C18 column; 0.5-min fractions were collected and assayed for radioactivity. In the lower panel, Tyr *O*-sulfate standard was derivatized and chromatographed under the same conditions and monitored for absorbance at 254 nm.

with M_r 340 000 HepG2 fibrinogen on polyacrylamide gels. When reduced, the constituent α-, β-, and γ-chain bands comigrated with their normal counterparts. These results show that rFbg was secreted as the normal six-chain molecule with a stoichiometry of $(\alpha\beta\gamma)_2$. Similarly, rFbgγ' showed identical α and β chains upon reduction, with the larger γ' chain migrating more slowly, as expected.

Functionally, the recombinant fibrinogens were active in both clotting and cross-linking. It is significant that the labeled recombinant fibrinogens not only were incorporated into the clot but also were cross-linked by the factor XIIIa transglutaminase activity. This indicates that the recombinant fibrinogens were aligned within the fibrin fibrils in the correct orientation, such that the carboxyl ends of the γ (or γ') chains in adjacent fibrin monomers were close enough to one another for cross-linking into dimers; similarly, the α chains were in the correct orientation to allow cross-linking into a high molecular weight polymer. Thus, by these criteria, the recombinant fibrinogens functioned like normal HepG2 fibrinogen.

An interesting structural difference seen between rFbg and rFbgγ' was the sulfation of the γ' chain. An early publication (Jevons, 1963) reported the presence of Tyr *O*-sulfate in human fibrin, which was later localized to the β chain (Liu et al., 1985), on the basis of its mobility on gels. A reexamination of these findings (Hortin, 1989) suggested that the sulfated chain may be the γ' variant, on the basis of its mobility and protease resistance. The present report demonstrates that the γ' chain is indeed sulfated, rather than the closely migrating β chain.

Pronase hydrolysis of the sulfated chain in human fibrinogen from HepG2 cells showed that the sulfation occurred on Tyr residues (Liu et al., 1985; Hortin, 1989). In the BHK cell expression system, the vast majority of the sulfation was also found on Tyr residues, demonstrating that the expression system performs this posttranslational modification correctly. In rat fibrinogen, one Tyr at position 418 is present in the γ' chain which is absent in the γ chain; this is thought to be the sulfated residue (Hirose et al., 1988). Similarly, the human γ' chain also contains Tyr-418; however, an additional Tyr is also present at position 422 (Figure 6). Tyr-418 follows the consensus pattern for sulfated Tyr residues (Huttner, 1988): an acidic amino acid at position −1 (Glu-417) with at least three acidic amino acids from −5 to +5 (Glu-415, Glu-417, Asp-419) and not more than one basic amino acid from −5 to +5 (none present); the presence of turn-inducing amino acids from −7 to −2 and from +1 to +7 (Pro-413, Pro-423); less than three hydrophobic amino acids from −5 to +5 (Leu-421); and an absence of disulfide-bonding Cys residues or N-linked glycosylation sites from −7 to +7. In contrast, Tyr-422 lacked an acidic amino acid at position −1 (Leu-421) but had five acidic amino acids from −5 to +5 (Glu-417, Asp-419, Glu-424, Asp-425, Asp-426), had one turn-inducing amino acid from +1 to +7 (Pro-423) but lacked one from −7 to −2, had less than three hydrophobic amino acids from −5 to +5 (Leu-421, Leu-427), and lacked disulfide-bonded Cys residues or N-linked glycosylation sites from −7 to +7. On the basis of this analysis, and by analogy with the rat γ' chain, Tyr-418 is probably the sulfated residue. Further biochemical characterization of the Tyr *O*-sulfate, however, is necessary. It was not possible to determine the stoichiometry of sulfation, since the specific activities of the cysteine and sulfate pools within the cell are not known. The determination of the stoichiometry of sulfation will require the purification of rFbgγ' and the biochemical analysis of the sulfated Tyr residues. One additional possibility is that the γ'-chain 20 amino acid carboxyl extension is not sulfated itself but merely directs sulfation to another part of the chain. However, the rapid and quantitative release of the sulfated Tyr by carboxypeptidase Y suggests that the sulfated residues were near the carboxyl terminus.

The role of the sulfated Tyr is completely unknown, since the role of the γ' chain is unknown. It is apparent that the γ'-chain extension contributes seven extra negatively charged amino acids in Glu and Asp residues, and possibly one or two additional negative charges in sulfated Tyr residues. The effect of these charges on the fibrinogen molecule is puzzling. It is clear from the present data that they do not prevent incorporation into a fibrin clot, nor do they prevent cross-linking of the γ' chains by factor XIIIa. Previous studies using heterodimeric γ'-containing fibrinogen $(\alpha\beta\gamma)(\alpha\beta\gamma')$ indicated that these molecules have reduced platelet binding and aggregate platelets less effectively (Peerschke et al., 1986). In addition, intracellular fibrinogen stored in platelet α granules lacks the γ' chain (Francis et al., 1984; Mosesson et al., 1984). However, these findings only illustrate roles in which γ'-

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                                  408
Rat gamma chain      NH2---Val-Gly-Asp-Met-COOH

Rat gamma' chain     NH2---Val-Ser-Val-Glu-His-Glu-Val-Asp-Val-Glu-Tyr-Pro-COOH

Human gamma chain    NH2---Ala-Gly-Asp-Val-COOH

Human gamma' chain   NH2---Val-Arg-Pro-Glu-His-Pro-Ala-Glu-Thr-Glu-Tyr-Asp Ser-Leu-Tyr-Pro-Glu-Asp-Asp-Leu-COOH
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FIGURE 6: Comparison of γ- and γ'-chain carboxyl termini from rat and human fibrinogens. Amino acid sequences for the carboxyl termini of rat and human γ and γ' chains are shown in alignment. The putative Tyr-418 sulfation site in the rat γ' chain is underlined, as is the corresponding site in the human γ' chain and the unique Tyr at position 422.

containing fibrinogen does not participate; the expression system shown in this report should prove useful in the elucidation of its true role in hemostasis.

ACKNOWLEDGMENTS

We thank Marie Beltran for technical assistance with the expression of rFbgγ'. We also thank Dr. Don Foster for providing the α_2-antiplasmin expression vector. We express our sincere appreciation to Dr. Stephen Conroy for his assistance with the Tyr *O*-sulfate analysis and to Dr. Ming-Cheh Liu for his generous gift of the Tyr *O*-sulfate standard.

Registry No. Tyr, 60-18-4.

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