Characterization of Purified Recombinant Fibrinogen: Partial Phosphorylation of Fibrinopeptide A†

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ABSTRACT: Human fibrinogen has been expressed in Chinese hamster ovary (CHO) cells using a novel two-step procedure which permits efficient synthesis of engineered variant fibrinogens. CHO cells secreting recombinant fibrinogen were grown in roller bottles and maintained in serum-free media for several months. Recombinant protein was purified from media containing 2–4 μg/mL fibrinogen using protamine–Sepharose chromatography. Recombinant fibrinogen was identical to plasma fibrinogen when examined on Coomassie-stained SDS gels run under reducing conditions, and on SDS gels when run under nonreducing conditions after partial or complete plasmin degradation, indicating normal chain assembly, disulfide bond formation, and overall protein conformation. Thrombin digestion of purified fibrinogen led to clot formation with release of normal fibrinopeptides, as identified by HPLC. Fibrinopeptide A released from recombinant fibrinogen was partially phosphorylated (22%), similar to the degree of phosphorylation found for human plasma fibrinogen (20–25%), indicating that partial phosphorylation in inherent in fibrinogen synthesis.

Fibrinogen is a soluble plasma protein comprised of a dimer of 3 polypeptides, Aα, Bβ, and γ, held together by 29 disulfide bonds. During clot formation, fibrinogen is converted to insoluble fibrin as a consequence of thrombin-catalyzed removal of fibrinopeptides A (FpA)[1] and B (FpB) from the amino termini of the Aα and Bβ chains, respectively. The primary structure of human fibrinogen was originally determined by amino acid sequence analysis of the protein (Doolittle et al., 1979; Henschen et al., 1980; Watt et al., 1979), and subsequently confirmed by cDNA sequence determination (Chung et al., 1983a,b; Rixon et al., 1983; Kant et al., 1983). The three polypeptides are encoded by separate cDNAs transcribed from three genes located in a 45-kb segment of chromosome 4 (Kant et al., 1984; Henry et al., 1984). Human fibrinogen is modified by several posttranslational events including glycosylation of the Bβ (Watt et al., 1979) and γ chains (Iwanaga et al., 1968) and partial phosphorylation of FpA at Aα Ser^3 (Blombäck et al., 1962).

Our laboratory has been using protein engineering to study the relationship of primary structure to fibrinogen function. For these studies, we have previously expressed each fibrinogen chain individually, as well as domains from within these chains, in *Escherichia coli* (Lord, 1985; Bolyard & Lord, 1988, 1989, 1991; Lord & Fowlkes, 1989; Lord et al., 1990). Engineered varient proteins expressed in these procaryotic systems have provided new information on domains important for fibrinogen functions (Lord & Fowlkes, 1989; Lord et al., 1990; Bolyard & Lord, 1991). In order to analyze altered domains in the context of the intact functional fibrinogen molecule, we have now expressed recombinant fibrinogen in Chinese hamster ovary (CHO) cells. The synthesis of biologically active recombinant human fibrinogen was first reported in 1989 (Farrell et al., 1989). In this expression system, recently described in detail (Farrell et al., 1991), two plasmid vectors, one containing the cDNAs for Aα and γ and one containing the Bβ cDNA and a selectable marker, DHFR, were cotransfected into BHK cells, and stable clones expressing fibrinogen were isolated.

Expression of fibrinogen has also been demonstrated in COS1 cells. Stable expression was reported by Roy et al. (1991), who contransfected COS1 cells with three plasmids each containing one fibrinogen cDNA in tandem with the selection marker neo. Transient expression of biologically active fibrinogen in COS1 cells transfected with three plasmids, each with a fibrinogen cDNA, has also been described (Hartwig & Danishefsky, 1991). Additional data obtained from transfection with single cDNAs or mixtures of two cDNAs were also used to study the pathway for assembly and secretion of fibrinogen in both COS1 expression systems. Roy et al. (1991) reported that cells transfected with one or two cDNAs expressed but did not secrete individual chains. In contrast, Hartwig and Danishefsky reported secretion of free Aα, free γ, and an Aα·γ_2 complex.

The expression system described here differs from those previously reported in that we have developed a two-step procedure which permits efficient synthesis of variant fibrinogens. Expression plasmids encoding Aα and γ were cotransfected with pRSVneo into CHO cells. Cells resistant to G418, a neomycin analog, were isolated, and lines synthesizing high levels of both Aα and γ chains were identified. These cells were found to secrete an Aα·γ complex. To obtain recombinant fibrinogen, one Aα·γ G418-resistant cell line was transfected with the Bβ expression plasmid and a second selection vector, pMSVhis. Colonies resistant to neomycin and histidinol were selected, and clonal lines expressing recombinant human fibrinogen were obtained. The purification and biochemical characterization of this recombinant protein are described here. Our results dem-

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[1] Abbreviations: BHK, baby hamster kidney; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HPLC, high-performance liquid chromatography; MLP, major late promoter; PCR, polymerase chain reaction; SFM, serum-free media; TBS, Tris-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine.

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onstrate that FpA in fibrinogen synthesized in CHO cells is phosphorylated to the same extent as FpA in plasma fibrinogen.

EXPERIMENTAL PROCEDURES

Materials. The mammalian expression vector (Berkner, 1988) was provided by Dr. K. Berkner, ZymoGenetics (Seattle, WA). Plasmids pHIβ2 (Chung et al., 1983b) and pHIγ2 (Chung et al., 1983a), containing cDNAs for the Bβ and γ chains of human fibrinogen, were provided by Dr. D. Chung, University of Washington, Seattle, WA. pRSVneo (Gorman et al., 1983) was obtained from Dr. B. Howard, NCI, Bethesda, MD, and pMSVhis (Hartman & Mulligan, 1988) was provided by Dr. R. Mulligan, Whitehead Institute, Cambridge, MA. Unless otherwise specified, all reagents were from Sigma.

Construction of Expression Vectors. The cDNAs encoding the three fibrinogen chains were cloned into an expression vector, p284, which was constructed by insertion of a multiple cloning site linker (AATTGTCGACCGCGGCCCCGGGCG-GCCGC) into the *Eco*RI site of p272 (Pei et al., 1991). This vector, diagrammed in Figure 1, contains the major late promoter (MLP), the tripartite leader sequence, and a 5′-splice site from adenovirus 2, a 3′-splice site from a mouse IgG gene, and the polyadenylation site from SV40 (K. Berkner, personal communication). Construction of the vector for Bβ-chain expression is outlined in Figure 1. The Bβ cDNA was obtained by PCR with *taq* polymerase (Promega) using the plasmid pHIβ2 (Chung et al., 1983b) as template, the deoxyoligonucleotides 5′-CCCCGTCGACATGAAAAGA-ATGGTTTCGTGGAGCTTCCACAAACTTAAAACCAT -3′ and 5′-TTGTCACATACAGAAGAG-3′ as the primer pair, and conditions previously described (Graham et al., 1989). The genomic Bβ sequence contains three in-frame initiation ATG codons at positions −30, −27, and −16 (Chung et al., 1983b). The amplified 1525 bp fragment contained the three potential initiation codons, the first two of which are absent in pHIβ2. The actual start site is unknown. The amplified cDNA was cleaved in *Sal*I and *Sna*BI and cloned into p284 cleaved with *Sal*I and *Sma*I to give the expression plasmid pMLP-Bβ. Cloning procedures were as previously described (Lord et al., 1990). Enzymes were purchased from New England Biolabs and Promega. The cloned Bβ cDNA in the expression vector was sequenced using Sequenase V2.0 as described by the manufacturer (U.S. Biochemicals). No inadvertent changes arose as a result of these cloning procedures.

The Aα cDNA was obtained from plasmid p549.12, a plasmid essentially identical to the previously described p115.6 (Lord, 1985), but containing an additional adenosine residue. Resequencing of the Aα cDNA showed that the clone did not contain the sequence previously reported for bases 540–555 (TCTGCAAAAAAATGTT, Kant et al., 1983). The sequence of the cDNA was TCTGCAAAAAATGTT. The missing adenosine residue was inserted by oligonucleotide-directed mutagenesis (Horton & Lord, 1986) using an M13 Aα subclone as template and the deoxyoligonucleotide 5′-CAACTGGGCCCTAACATTTTTTTGCAG-3′ as the mutagenic primer. The corrected cDNA segment was subcloned as a *Bgl*I/*Kpn*I fragment into p115.6 prepared by methylation with *Fnu*DI methylase followed by cleavage with *Bgl*I and *Kpn*I, to produce the plasmid p549.12. The Aα expression vector was constructed by insertion of a *Pvu*II/*Ssp*I Aα cDNA fragment from p549.12 into p284 cleaved with *Sma*I. This vector (pMLP-Aα) was completely sequenced, and no inadvertent changes were found. The cDNA encodes Ser^{47}, Thr^{296}, and Ala^{312} at the three known polymorphic codons (Ebert, 1991).

The γ cDNA was isolated from pHIγ2 (Chung et al., 1983a) as a *Sac*I/*Ssp*I fragment, the *Sac*I blunted with T4 DNA polymerase (U.S. Biochemicals), and the fragment ligated into p284 which had been cleaved with *Sma*I. The orientation of the γ cDNA insert was established by restriction enzyme analysis. CHO cells transfected with this vector, pMLP-γ, synthesized an immunoreactive protein which comigrated with plasma fibrinogen γ chain on SDS–PAGE. However, subsequent Western blot analysis using the monoclonal antibody 4A5 (Matsueda et al., 1988) demonstrated that this recombinant chain lacked the 4A5 epitope. We therefore sequenced the γ cDNA insert in pMLP-γ and found that this cloned cDNA was truncated at amino acid 403, instead of the normal γ411. The cDNA insert was truncated and fused to vector sequences which maintained an open reading frame for three additional amino acids such that the C-terminus of this chain was ---HHLGPQF instead of the normal γ sequence—HHLGGAKQAGDV; no other inadvertent changes were found.

DNA Transfections and Cell Culture. CHO cells (ATCC CCL-61), grown in DMEM/F12 supplemented with 5% bovine calf serum (HyClone Laboratories)/5% Nu-serum (Collaborative Research)/10 IU/mL penicillin/10 μg/mL streptomycin, were used as the host for all transfections. Plasmid DNA was prepared by scale-up of the procedures used for preparing plasmids for DNA sequence analysis (Lord & Fowlkes, 1989). CHO cells for transfection were plated at 1:10 dilutions into 100-mm plates (Falcon) and grown overnight. Fresh media were added 1 h before transfection. Transfections were by standard calcium phosphate coprecipitation (Kingston et al., 1989) using a total of 11 μg of DNA and a 10:1 ratio of expression vector to selection vector. Cells were incubated with precipitated DNA for 4 h, the media removed, and the cells shocked with 10% glycerol for 2–3 min. Cells were grown for 24 h in normal media and split 1:10 into 100-mm plates. Once cells were attached to the plates, G418 and/or histidinol were added for clonal selection. Cells transfected with pRSVneo were selected with 400 μg/mL G418, a neomycin analog (GIBCO Bethesda Research Laboratories). Cells transfected with pMSVhis were selected with 2.5 mM histidinol (Aldrich). G418-resistant cells transfected with pMSVhis were selected and grown in both G418 and histidinol. Individual colonies were picked from selection plates, grown to confluence, and tested for fibrinogen chain synthesis. Positive cell lines were subcloned by infinite dilution. Large-scale cell growth in roller bottles was as previously described (Pei et al., 1991) with the exception that aprotinin (10 U/mL; Calbiochem) was added to serum-free media during cell growth, and no other inhibitors were added to the media after harvesting.

Immunologic Analysis of Cell Lysates and Media. For Western blot analysis of cell lysates, healthy resistant colonies were grown to confluence in 35-mm plates. Cells were washed with cold phosphate-buffered saline, lysed by addition of Laemmli sample buffer (Laemmli, 1970), and repeatedly freeze-thawed. For analysis of secreted proteins, serum-free medium was added to confluent CHO cells, and 3–7 days later, aliquots were removed and mixed with Laemmli buffer. Samples were run on polyacrylamide gels and blotted onto 0.20-μm nitrocellulose (Fisher). After incubation in 0.5% gelatin in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20), blots were incubated with an antibody specific for either human fibrinogen or one chain of human fibrinogen. Polyclonal rabbit anti-human fibrinogen was purchased from DAKO (Carpinteria, CA). Monoclonal antibodies to the Bβ chain and an internal region of the Aα

chain (9C3) were from Dr. J. Shainoff (University of Michigan, Ann Arbor, MI) and Dr. A. Budzynski (Temple University, Philadelphia, PA), respectively. The polyclonal antibody to γ chain (2625) was from Dr. E. Plow (Scripps Research Foundation, LaJolla, CA). Alkaline phosphatase conjugated secondary antibody was either goat anti-rabbit (Pierce) or goat anti-mouse (Promega). Blots were developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as described (Harlow & Lane, 1988). ELISA assays of conditioned media were as previously described (Snouwaert et al., 1991) using a goat anti-human fibrinogen capture antibody (Cappel, Durham, NC) and a peroxidase-conjugated goat anti-human fibrinogen (Cappel) and TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for detection. Alternatively, detection was with the monoclonal antibody to the Bβ chain followed by a peroxidase-conjugated goat anti-mouse antibody (Cappel).

Protamine–Sepharose Chromatography. Protamine–Sepharose was prepared as described (Dempfle & Heene, 1987a). Fifty milliters of 1.5% protamine sulfate was coupled to 3 g of CNBr-activated Sepharose (Pharmacia—LKB) for 2 h at room temperature. Approximately 21 mg of protamine/mL of resin was bound as measured by the difference in protamine concentration in solution before and after coupling. Resin was poured into a glass column (final dimension 1 × 11 cm) and extensively washed as described.

Five hundred milliliters of CHO-conditioned serum-free media was filtered through Whatman 1 filter paper to remove particulate material and loaded onto the column. A modification of a published procedure (Dempfle & Heene, 1987b) was developed using plasma fibrinogen (Grade L, KabiVitrum, Stockholm, Sweden). The resin was preequilibrated with buffer A1 (50 mM Tris-HCl, pH 7.3, 5 mM EDTA, and 5 mM ϵ-aminocaproic acid) and washed with several column volumes of this buffer after the sample was loaded. Buffer A2 (as above with 800 mM NaCl) was then pumped through the column followed by buffer B1 (200 mM sodium citrate/citric acid, pH 5.3). Contrary to Dempfle and Heene (1987b), fibrinogen was not eluted by this buffer. A large protein peak was eluted by buffer B1, and SDS–PAGE indicated that this material did not contain significant amounts of fibrinogen (data not shown). Citrate was removed by passing 100 mM sodium acetate, pH 5.3, over the column, and the bound fibrinogen was then eluted with 100 mM sodium acetate, pH 4.5. This material was neutralized immediately by the addition of 1 M Tris-HCl, pH 8, and dialyzed overnight against 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl (TBS).

Analysis of Purified Fibrinogen. Samples of purified recombinant fibrinogen were analyzed by SDS–PAGE on 7.5% gels. The thrombin-catalyzed release of FpA and FpB was measured by HPLC. Fibrinogen (0.2 mg/mL) in TBS was incubated with 5 units/mL human α-thrombin (provided by Dr. F. Church, University of North Carolina at Chapel Hill) at 37 °C for 1 h. The resulting clots were boiled for 3 min, and microfuged for 5 min to pellet the clot. The supernatant was injected into a C18 HPLC column and eluted by an acetonitrile gradient (Haverkate et al., 1986). Removal of phosphate from phosphorylated FpA was performed by digestion of the HPLC-purified peptide with calf intestinal alkaline phosphatase (10 units/mL, New England Biolabs) overnight at room temperature. Plasmin digests were as follows: 1 μL of plasmin (10 CTA U/mL in 50% glycerol; American National Red Cross) was added to 400 μL of fibrinogen (0.2 mg/mL) in TBS and incubated at room temperature. Aliquots were removed, added to an equal volume of nonreducing Laemmli buffer, and boiled for 3 min. Samples were run on 7.5% polyacrylamide gels and stained with Coomassie blue.

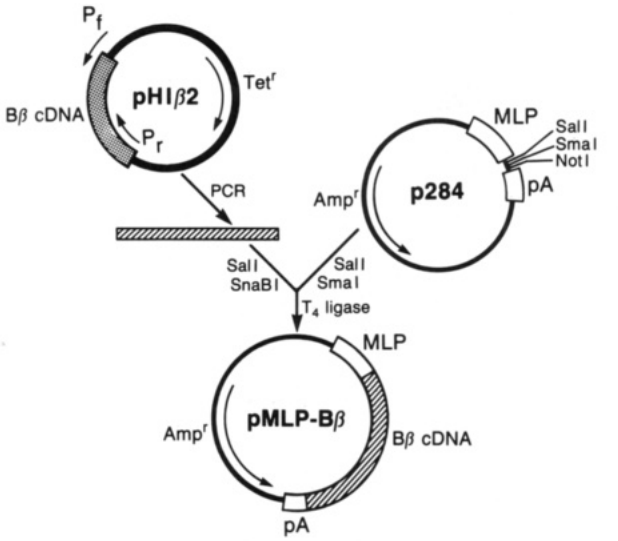

FIGURE 1: Construction of the Bβ-chain expression vector pMLP-Bβ.

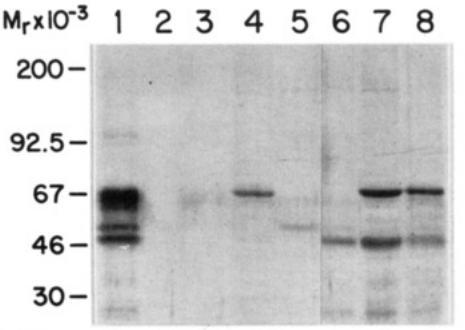

FIGURE 2: Western blot of CHO cell lysates. Reduced cell lysates were subjected to SDS–PAGE on 7.5% gels and blotted onto nitrocellulose. Primary antibodies were rabbit anti-human fibrinogen and anti-γ chain, secondary antibody was alkaline phosphatase linked goat anti-rabbit IgG. Lane 1, reduced Kabi fibrinogen (90 ng); lane 2, blank; lane 3, untransfected CHO; lane 4, CHO-Aα; lane 5, CHO-Bβ; lane 6, CHO-γ; lane 7, CHO-Aα·γ; lane 8, CHO-fibrinogen.

RESULTS

Expression of Recombinant Fibrinogen and Fibrinogen Chains. As described under Experimental Procedures, each fibrinogen chain cDNA was subcloned into the expression vector p284 downstream from the adenovirus 2 major late promoter (MLP). The construction of the Bβ-chain vector pMLP-Bβ is depicted in Figure 1. The cDNAs coding for Aα and γ were subcloned as blunt-end plasmid fragments into *Sma*I-cleaved p284, producing expression vectors pMLP-Aα and pMLP-γ. The cDNA inserts in each expression vector were sequenced and found to be normal with the exception of a short deletion in pMLP-γ, as described under Experimental Procedures.

CHO cells were transfected with pRSVneo and each individual chain vector (pMLP-Aα, or pMLP-Bβ, or pMLP-γ), or with both pMLP-Aα and pMLP-γ. Lysates of neo-resistant cells (CHO-Aα, CHO-Bβ, CHO-γ, and CHO-Aα·γ) were analyzed by Western blots developed with polyclonal antisera to human fibrinogen. As shown in Figure 2, lanes 4–6, lysates from cells containing individual chains expressed immunoreactive proteins which migrated at the same position as the corresponding chains in plasma fibrinogen (Figure 2, lane 1). Several neo-resistant CHO-Aα·γ cell lines were

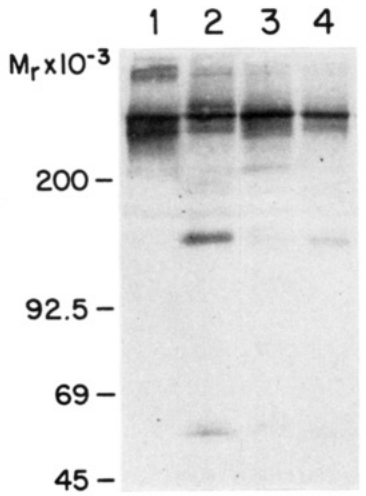

FIGURE 3: Western blot of conditioned serum-free media (SFM). Conditioned SFM (40 μL) samples in nonreducing sample buffer were run on a 3–12% gradient gel. Otherwise, conditions were as described in Figure 2. Lane 1, Kabi fibrinogen (90 ng); lane 2, HepG2; lanes 3 and 4, two CHO-fibrinogen clones. The lower band in lane 1 is a degraded form of fibrinogen commonly found in plasma fibrinogen preparations.

isolated, and some of these expressed high levels of both Aα and γ chains, as shown in Figure 2, lane 7. One cell line which expressed high levels of both Aα and γ chains was cotransfected with pMLP-Bβ and pMSVhis. Colonies were selected for resistance to histidinol, as well as G418. ELISA analysis of culture media, with a monoclonal antibody to the Bβ chain, demonstrated that most (23 of 24 tested) of these colonies secreted fibrinogen. Western blot analysis of cell lysates clearly demonstrated the presence of Aα and γ chains, as seen in Figure 2, lane 8. It is clear that these cells lysates contained more Aα and γ chains than Bβ chains, consistent with previous observations that only Aα and γ chains accumulate in the endoplasmic reticulum of HepG2 cells, a hepatoma line which secretes fibrinogen (Roy et al., 1990).

Using antisera to fibrinogen, ELISA analysis of the culture media from representatives of all five cell lines (CHO-Aα, CHO-Bβ, CHO-γ, CHO-Aα·γ, and CHO-fibrinogen) demonstrated that immunoreactive material was secreted from cells transfected with γ chain alone, with both Aα and γ chains, and with all three chains. The presence of recombinant protein in the media of CHO-γ and CHO-Aα·γ probably is not the result of cell lysis as we found no recombinant protein in the media of CHO-Aα and CHO-Bβ cells grown under similar conditions, and these latter two cell lines had intracellular accumulation of Aα and Bβ chains, respectively. Quantitative ELISA analysis, using polyclonal antisera to fibrinogen, indicated that the relative levels of secretion were 1:6:40 for CHO-γ/CHO-Aα·γ/CHO-fibrinogen. Western blot analysis of culture media samples from CHO-fibrinogen cells demonstrated the presence of three bands with relative concentrations and electrophoretic mobilities equivalent to plasma fibrinogen (data not shown). Analysis of aliquots of these media prepared under nonreducing conditions demonstrated the presence of a 340-kDa band with mobility identical to nonreduced plasma fibrinogen, as shown in Figure 3. Smaller, less prominent, cross-reacting bands were also seen in the media samples, similar to those seen in media samples from HepG2 cells (Figure 3, lane 2).

As shown in Figure 4, Western blot analysis of media from CHO-γ (Figure 4A) and CHO-Aα·γ (Figure 4B) cells demonstrated that γ chains alone were found in the culture media of CHO-γ cells and that both Aα and γ chains are found in the media of CHO-Aα·γ cells (Figure 4B, lane 2).

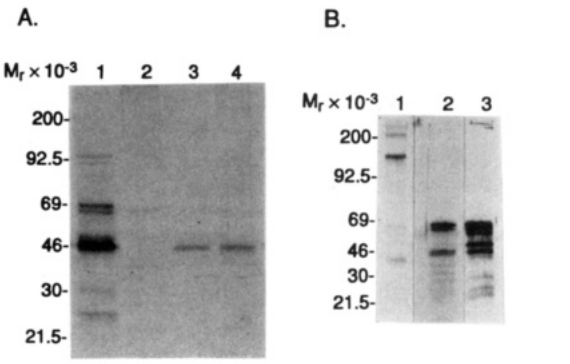

FIGURE 4: Western blot of conditioned serum-free media (SFM) secreted by CHO-γ and CHO-Aα·γ cells. Conditioned SFM were mixed with an equal volume of reducing or nonreducing buffer and run on 7.5% SDS–PAGE. (A) Lane 1, Kabi fibrinogen (reduced, 90 ng); lane 2, CHO; lanes 3 and 4, two CHO-γ clones (reduced). (B) Lane 1, CHO-Aα·γ (nonreduced); lane 2, CHO-Aα·γ (reduced); lane 3, Kabi fibrinogen (reduced). Primary antibody for (A) was anti-γ chain. Primary antibody for (B) was anti-fibrinogen.

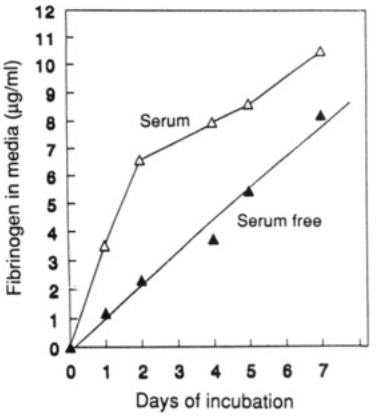

FIGURE 5: Accumulation of fibrinogen in CHO-fibrinogen conditioned media. Confluent 100-mm plates of CHO-fibrinogen cells were incubated with 10 mL of DMEM/F12 with or without 5% Nuserum/5% calf serum. At time intervals, aliquots were taken, and the fibrinogen concentration was measured by ELISA.

When the latter samples were prepared under nonreducing conditions (Figure 4B, lane 1), an immunoreactive complex with a relative molecular weight of approximately 150 000 was found. Similar blots developed with a polyclonal antibody to γ chain or a monoclonal antibody to Aα chain revealed that this 150-kDa band contained both Aα and γ immunoreactive material (data not shown).

Characterization of Recombinant Fibrinogen Secretion. One cell line which secreted high levels of fibrinogen was subcloned by dilution procedures. Subcloned cell lines were then grown to confluence in 100-mm dishes in the presence of complete selection medium, as described under Experimental Procedures. Cells were maintained in media with and without serum, and fibrinogen secretion was quantitated by ELISA analysis. Immunoreactive material accumulated in the media, as shown in Figure 5, reaching a level of 8 μg/mL after 7 days in the absence of serum. The initial rate of secretion was greater in serum-containing media, although after 2 days the rates became similar. Recombinant fibrinogen was harvested from serum-free media to simplify the purification and to prevent protein degradation that might occur from trace amounts of proteinases present in serum. Western blot analysis of serum-free media samples removed at several time points indicated that the accumulating protein remained essentially intact during this time (data not shown).

Purification of Recombinant Fibrinogen. To obtain sufficient quantities for biological characterization of purified recombinant fibrinogen, CHO cells were grown in roller bottles

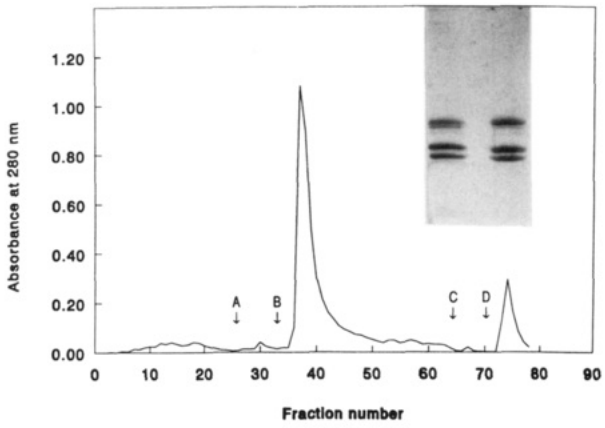

FIGURE 6: Purification of recombinant fibrinogen by protamine–Sepharose chromatography. CHO-fibrinogen conditioned SFM (500 mL) was loaded onto a 1 × 11 cm protamine–Sepharose column. The column was then washed with 50 mM Tris-HCl, pH 7.3, and 5 mM ϵ-aminocaproic acid, with this buffer and 800 mM NaCl (A), with 200 mM citric acid/sodium citrate, pH 5.3 (B), and finally with 100 mM sodium acetate, pH 5.3 (C). The recombinant fibrinogen was eluted with 100 mM sodium acetate, pH 4.5 (D). Inset: Coomassie blue stained 7.5% SDS–polyacrylamide gel of Kabi fibrinogen (left) and purified recombinant fibrinogen (right) run under reducing conditions.

with microcarrier beads. Cells were maintained in serum-free media without G418 and histidinol. Fibrinogen secretion was monitored by ELISA. Typically, 100 mL of media was removed every third day and replaced with 100 mL of fresh media. Recombinant fibrinogen was purified from media by chromatography on protamine–Sepharose. In initial experiments, protein was concentrated by precipitation with 50% saturated ammonium sulfate prior to chromatography. Subsequently, we found that direct application of the media was more efficient and resulted in less degradation of the recombinant fibrinogen. The elution profile of a sample chromatograph is presented in Figure 6. Little material was eluted by high salt; 200 mM citrate, pH 5.3, eluted a large peak; however, this material contained little or no fibrinogen as indicated by SDS–PAGE. Neither recombinant fibrinogen nor Kabi fibrinogen in parallel experiments was eluted by this buffer in contrast to a previous report (Dempfle & Heene, 1984b). This discrepency may be due to different preparations of protamine (O. Gorkun, personal communication). Recombinant fibrinogen or Kabi fibrinogen was, however, eluted by sodium acetate buffer, pH 4.5. Purified recombinant fibrinogen was analyzed by SDS–PAGE gels stained with Coomassie blue, and as shown in the inset in Figure 6, the purified recombinant material migrated in a manner indistinguishable from plasma fibrinogen.

Functional Analysis of Purified Recombinant Fibrinogen. Purified recombinant fibrinogen formed a clot when incubated with human α-thrombin. The clotted sample ([fibrinogen] = 0.065 mg/mL) formed a gel of sufficient solidity that the gel remained intact in an inverted cuvette. The thrombin-catalyzed release of FpA and FpB was demonstrated by HPLC analysis (Figure 7). The HPLC profiles were similar to those seen with plasma fibrinogen, including the presence of the peak FpA(P), previously shown to by FpA in which Ser^3 is phosphorylated (Blombäck et al., 1962). The identity of this peak was confirmed by collecting this peak, treating it overnight with alkaline phosphatase, and reinjecting into the HPLC. As shown in Figure 8C, the phosphatase-treated peptide eluted at the position of normal FpA. We quantitated the released fibrinopeptides and found that after incubation with 5 units/mL thrombin for 60 min at 37 °C, the fibrinopeptide peak areas for recombinant fibrinogen were identical to those found with plasma fibrinogen incubated under the same conditions.

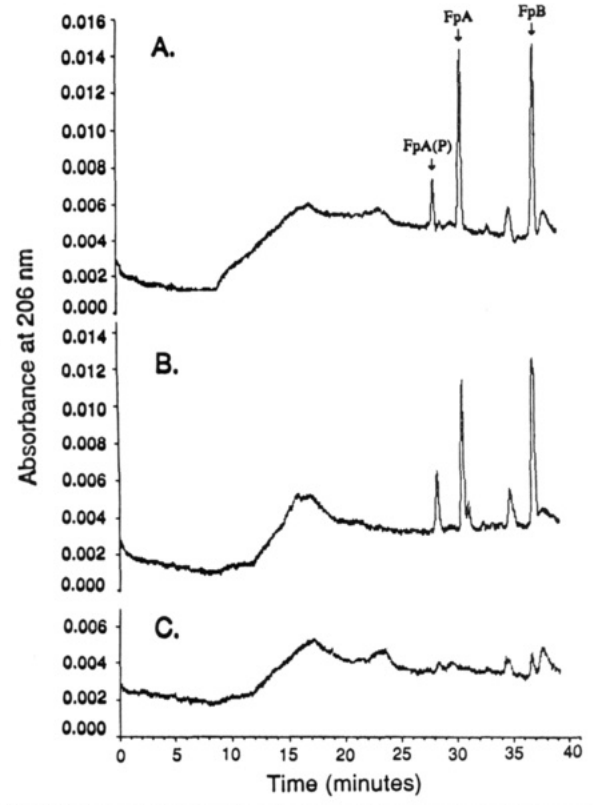

FIGURE 7: Thrombin-catalyzed fibrinopeptide release from recombinant fibrinogen. Recombinant fibrinogen (0.2 mg/mL) was digested with 5 units/mL human thrombin for 1 h at 37 °C. Fibrin was precipitated by boiling and pelleted by centrifugation. The fibrinopeptides present in the supernatant were eluted from a C18 reverse-phase HPLC column by an acetonitrile gradient. (A) Thrombin-digested recombinant fibrinogen; (B) thrombin-digested Kabi fibrinogen; (C) undigested recombinant fibrinogen. FpA, fibrinopeptide A; FpA(P), phosphoserine fibrinopeptide A; FpB, fibrinopeptide B.

Plasmin degradation of purified recombinant fibrinogen was similar to that seen with purified plasma fibrinogen. Plasmin cleaves fibrinogen sequentially into well-characterized fragments (Takagi & Doolittle, 1975). As shown in Figure 9, fragments X, Y, D, and E were formed in the same sequence with purified recombinant fibrinogen and plasma fibrinogen. After a 5-min incubation, only fragment X was seen for both recombinant and plasma fibrinogens. With longer incubations, X decreased as Y increased, and finally predominantly fragments D and E were visible. The similarity in these plasmin digest patterns indicated that recombinant fibrinogen is a dimer of three polypeptide chains that are covalently linked in the same manner as plasma fibrinogen.

DISCUSSION

Previously, we have demonstrated the utility of oligonucleotide-directed alterations of fibrinogen domains expressed in procaryotes to examine biological functions associated with these domains. The expression system and purification protocols described here demonstrate that similar studies with intact fibrinogen are feasible with the advantage that modification can now be examined in the context of the complete molecule. Fibrinogen-secreting CHO cells were produced by a two-step approach in which cDNAs coding for the Aα and γ chains were initially introduced. A cell line expressing high levels of both chains was identified and transfected with cDNA coding for the Bβ chain. The introduction of the Bβ chain into CHO-Aα·γ cells was very efficient (23 or 24 tested were

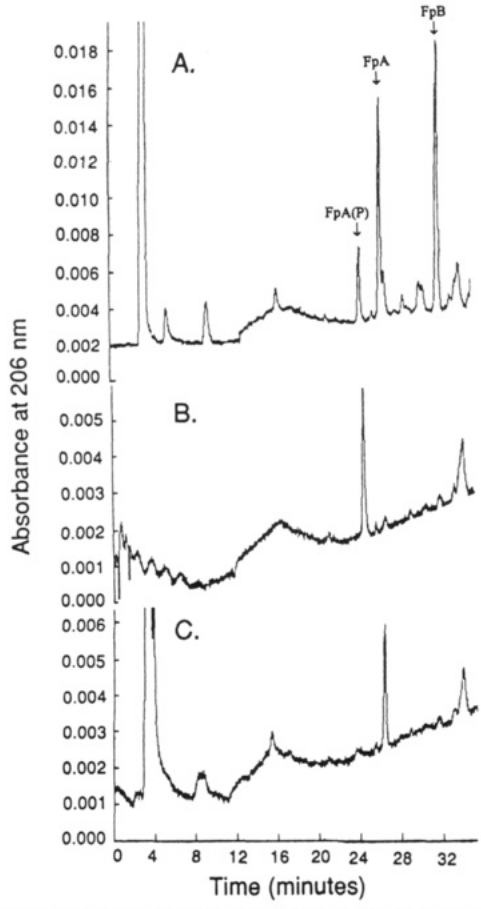

FIGURE 8: Dephosphorylation of FpA(P) from recombinant fibrinogen. The FpA(P) peak was collected from HPLC of a thrombin digest of 0.3 mg of recombinant fibrinogen. The peptide was lyophilized and rehydrated with 200 μL of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$, and half was digested with alkaline phosphatase. (A) Thrombin-digested Kabi fibrinogen; (B) untreated FpA(P); (C) alkaline phosphatase-treated FpA(P).

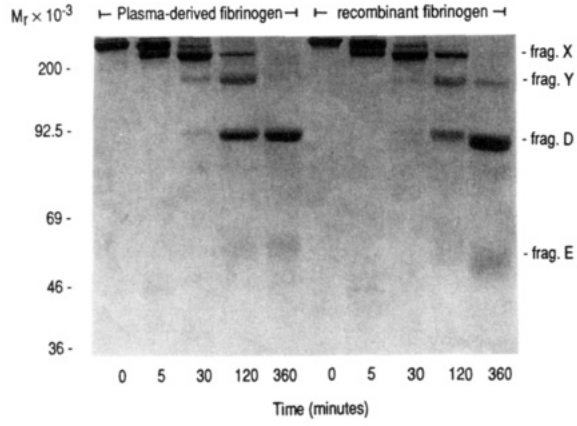

FIGURE 9: Plasmin digestion of recombinant and plasma-derived fibrinogen. Eighty micrograms of recombinant or Kabi fibrinogen in TBS was incubated with 0.01 CTA unit of plasmin at room temperature. Eight-microgram aliquots were then added to an equal volume of nonreducing buffer, run on 7.5% SDS–polyacrylamide gels, and stained with Coomassie blue. The resulting fragments (X, Y, D, and E) are labeled.

positive), indicating that this procedure will accelerate the process of producing clones that secrete fibrinogens with variant sequences. We have already used these cells to successfully generate a recombinant fibrinogen with an altered Bβ chain (C. G. Binnie and S. T. Lord, unpublished data). The ELISA assay allowed for simple and rapid screening of media with or without serum for fibrinogen immunoreactive material at an early stage during selection. Western blots gave more specific information regarding the species in solution but required serum-free media as the high serum albumin levels interfered with gel electrophoresis.

An additional advantage of this expression system is that the CHO cells secreting recombinant fibrinogen can be maintained for extended periods in the absence of serum. Our results demonstrate that the secreted fibrinogen is stable in the media for several days at 37 °C. The level of expression from CHO-fibrinogen cells has also been remarkably constant. CHO-fibrinogen cells that have been in serum-free media for 3 months without G418 or histidinol continue to express fibrinogen levels similar to those found when first plated. Using roller bottles and microcarrier beads, these cultured cells produced milligram quantities of recombinant fibrinogen. This level of expression is sufficient to study normal fibrinogen and engineered variant fibrinogen molecules and their conversion to fibrin, by techniques such as light scattering, HPLC, and clotting assays.

Purification of recombinant fibrinogen from conditioned serum-free media (SFM) was achieved by protamine–Sepharose chromatography. Recombinant fibrinogen behaved identically to plasma-derived fibrinogen and was eluted by pH 4.5 sodium acetate buffer. The posttranslational processing of the recombinant fibrinogen was similar to that seen in plasma-derived fibrinogen. SDS–PAGE analysis demonstrated that the individual chains had the same mobility as found with plasma fibrinogen, indicating the presence of carbohydrate on the Bβ and γ chains. We also found that glycosidase F treatment of recombinant fibrinogen increased the SDS–PAGE mobility of Bβ and γ chains, but not Aα chains, identically to plasma fibrinogen (data not shown). Plasmin digest patterns with recombinant fibrinogen were consistent with the normal disulfide bonds between the six chains. Fibrinopeptides A and B eluted on HPLC at the normal positions, indicating that the signal peptides present on each chain had been removed correctly. Finally, fibrinopeptide A was partially phosphorylated to a degree similar to that seen in plasma-derived fibrinogen. Thus, this analysis of purified recombinant fibrinogen structure is completely consistent with data obtained for plasma fibrinogen.

Human FpA is partially phosphorylated at Ser^3 (Blombäck et al., 1962). The degree of FpA phosphorylation observed in recombinant fibrinogen produced by CHO cells (22%) is within the range (20–25%) reported for purified human plasma fibrinogen (Blombäck et al., 1966). This observation is interesting as it indicates that the extent of phosphorylation in circulating fibrinogen is the result of partial phosphorylation during synthesis and is not a consequence of instability of phosphoserine in plasma. This indicates that FpA does not result from dephosphorylation of FpA(P) as previously postulated (Blombäck et al., 1962) but that only about one-fourth of the Aα chains are ever phosphorylated in the first place. This contrasts with canine fibrinogen, which is predominantly phosphorylated on the FpA and which is synthesized almost exclusively in the phosphorylated form (Kudryk et al., 1982). The role of the phosphate group in clotting has not been ascertained, but the occurrence of sulfate groups in the fibrinopeptide B of other mammalian species (Chang, 1983) points to the importance of negatively charged residues in fibrinopeptides. Similar correct posttranslational modification of recombinant fibrinogen has been reported by Farrell et al. (1991), who show that γ' chains are sulfated by BHK cells.

Characterization of the CHO cells transfected with one or two expression vectors demonstrated that CHO-γ and CHO-Aα·γ secrete recombinant protein into the media. Further-

more, we demonstrated by Western blot analysis with a monoclonal antibody to Aα and a polyclonal antibody to γ chain that the complex found in the media of CHO-Aα·γ cells contained both Aα and γ chains. These results are similar to those observed by Hartwig and Danishefsky (1991), who reported secretion from COS1 cells transfected with Aα, γ, or Aα and γ expression vectors and assayed 48 h later. They detected secreted protein by immunoprecipitation of in vivo radiolabeled material, which is probably more sensitive than the Western blot assays used in our experiments. That is, the method of detection may be the basis for the differences between Aα secretion detected in COS1 cells, and not detected in CHO cells. Our results also parallel those of Hartwig and Danishefsky (1991) in that we saw more Aα·γ complex secretion than γ-chain secretion when we examined media from CHO-Aα·γ and CHO-γ cells, respectively. In contrast, Roy et al. (1991), who also detected secreted protein by immunoprecipitation of in vivo radiolabeled material, found no secretion from COS1 cells after transfection and selection with G418, except when all three chains were present. In CHO-fibrinogen cells, the level of Bβ-chain accumulation, as indicated by band intensity on Western blot, was much lower than that for the other two chains, which is in agreement with Yu et al. (1984) and Roy et al. (1990), who found that the Bβ chain does not accumulate in HepG2 cells. We found that Bβ chains do accumulate in CHO cells when only this cDNA is present, as has been observed previously with COS1 cells (Danishefsky et al., 1990; Roy et al., 1991).

As described under Experimental Procedures, the recombinant fibrinogen synthesized here has a small alteration in the C-terminus of the γ chain. During characterization of this protein, we found that the fibrin gel formed with α-thrombin and this recombinant fibrinogen was slightly different from that formed with purified plasma fibrinogen. The recombinant fibrin gel appeared less rigid when disturbed by shaking; the plasma gel had more elasticity, returning to its original form rapidly after mechanical disturbance. These differences may reflect the variant nature of the recombinant γ-chain C-terminus, and we plan to pursue this question. We also plan to examine this variant molecule in assays for platelet aggregation and factor XIII cross-linking, functions which have been associated with this segment. We are currently preparing cell lines to synthesize recombinant fibrinogen with a completely normal sequence to use as controls for analysis of this variant protein. We are also preparing novel variant fibrinogens.

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