Plasma Factor XIII Binds Specifically to Fibrinogen Molecules Containing γ' Chains[†]

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ABSTRACT: The difference between peak 1 and peak 2 fibrinogen lies in their γ chains. Peak 1 molecules contain 2 γ_A chains; peak 2 molecules contain 1 γ_A and 1 γ' chain, the latter of which contains a 20 amino acid extension (γ' 408−427) replacing the carboxyl-terminal 4 amino acids of the γ_A chain (γ_A 408−411). While the existence of γ' chains in plasma fibrinogen molecules has been known for many years, their function remains unknown. When fibrinogen is purified from plasma, the factor XIII zymogen (A_2B_2) copurifies with it and is found only in the peak 2 fibrinogen when this fraction is separated from peak 1 fibrinogen by ion-exchange chromatography on DEAE-cellulose. Factor XIII alone applied to the same DEAE column elutes at a position between peak 1 and peak 2. When mixtures of peak 1 fibrinogen plus factor XIII or peak 2 fibrinogen plus factor XIII are applied to DEAE columns, the peak 1/factor XIII mixture elutes in two peaks, whereas the peak 2/factor XIII mixture elutes in the peak 2 fibrinogen position. Gel sieving on Superose 6 of peak 1/factor XIII mixtures results in two protein peaks, the first of which contains the fibrinogen. Most factor XIII activity elutes in the second peak with a small amount of activity emerging with the trailing end of the fibrinogen peak. Gel sieving of mixtures of peak 2 and factor XIII results in a single protein peak with all factor XIII activity emerging with the leading edge of the fibrinogen peak. The interaction between peak 2 fibrinogen and plasma factor XIII appears to be through binding to the B subunit of factor XIII since placental or platelet factor XIII (A_2), which does not contain B subunits, elutes independently from peak 2 fibrinogen on DEAE-cellulose chromatography. The results indicate that peak 2 fibrinogen γ' chains have a physiologically significant affinity for the B subunits of plasma factor XIII and that through this interaction fibrinogen serves as a carrier for the plasma zymogen in circulating blood.

Plasma factor XIII (protransglutaminase) is a noncovalent tetrameric zymogen complex composed of two pairs of polypeptide chains termed A and B, respectively (Schwartz et al., 1973; Chung et al., 1974; Cooke & Holbrook, 1974). Cleavage at position 37 of the A subunits by thrombin (Mikuni et al., 1973; Nakamura et al., 1974; Takagi & Doolittle, 1974) leads to formation of the active enzyme, factor XIIIa (transglutaminase), which requires Ca^{2+} for dissociation of the activated subunits (A'_2)[1] from B subunits and for full expression of its catalytic activity (Lorand & Konishi, 1964; Cooke, 1974; Curtis et al., 1974; Credo et al., 1978, 1981). Activation of factor XIII and the activity of factor XIIIa is promoted in the presence of fibrinogen (Credo et al., 1978, 1981; Janus et al., 1983; Greenberg et al., 1985a, 1987, 1988; Greenberg & Miraglia, 1985) and perhaps, more specifically, by fibrin (Janus et al., 1983; Greenberg et al., 1985a, 1987, 1988; Greenberg & Miraglia, 1985; Lewis et al., 1985; Naski et al., 1991; Procyk et al., 1993). Inhibition of fibrin polymerization with the peptide GPRP eliminates the enhancing effect of fibrin on activation of factor XIII (Greenberg & Miraglia, 1985; Lewis et al., 1985; Greenberg et al., 1986), suggesting that the fibrin effect is mediated through formation of a ternary complex among thrombin, fibrin, and factor XIII (Greenberg et al., 1987).

There is considerable evidence for more than one type of binding interaction between factor XIII and fibrin. Hornyak and Shafer (1992) reported that carbamylmethylated (CM) A'_2 chains bound more tightly to fibrin than did CM A_2 chains. Saturation of fibrin with plasma factor XIII (A_2B_2) did not affect CM A'_2 binding, suggesting that each form of the enzyme binds to a separate, nonoverlapping site on fibrin. Procyk et al. (1993) also found that A'_2 subunits bound to fibrin, but A_2 subunits did not. Greenberg et al. (1985b), however, suggested that there was a specific binding site for the factor XIII A_2 subunits on fibrin.

Evidence for a fibrinogen−factor XIII binding interaction and the nature of the binding reaction is incomplete and sometimes conflicting. Loewy et al. (1961a; Loewy, 1972) first suggested that plasma factor XIII existed in a complex with plasma fibrinogen, based upon the observations that factor XIII coprecipitated with plasma fibrinogen and migrated with it in immunoelectrophoretic or immunodiffusion experiments, and that its diffusion coefficient and

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; A_2B_2, plasma factor XIII; A_2, placental factor XIII or the catalytic subunits of plasma factor XIII; A'_2, activated catalytic subunits of factor XIII; B_2, noncatalytic subunits of plasma factor XIII.

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solubility properties changed after removal of fibrinogen by thermal denaturation. Consistent with this conclusion, Bannerjee and Mosesson (1975) showed by gel sieving analyses that the factor XIII zymogen (A_2B_2) in plasma or in a fibrinogen-rich ammonium sulfate fraction emerged in a position corresponding to a molecular weight of greater than 500 000. Following removal of the fibrinogen by thermal denaturation, the zymogen emerged in a position corresponding to the molecular weight of the factor XIII A_2B_2 tetramer. In contrast, Chung (1972) found no evidence for complex formation between factor XIII and fibrinogen, as assessed by gel filtration or ultracentrifugation. Greenberg and Shuman (1982) suggested on the basis of factor XIII binding to fibrinogen beads, that factor XIII bound specifically to fibrinogen by its A_2 subunits. Subsequently, Mary et al. (1987) confirmed that platelet factor XIII (A_2) chains bound specifically to fibrinogen, and thereby localized the binding site to the fibrinogen Aα and Bβ chains.

It has been known for many years that human fibrinogen can be resolved into two major peaks, peak 1 and peak 2 fibrinogen, by chromatography on DEAE-cellulose (Finlayson & Mosesson, 1963). Peak 2 fibrinogen amounts to 15% of the total plasma fibrinogen population and differs from peak 1 fibrinogen molecules only with respect to its γ chain composition (Mosesson et al., 1972; Wolfenstein-Todel & Mosesson, 1980). Peak 1 fibrinogen molecules contain 2 γ_A chains, which are comprised of 411 amino acids and which have a C-terminal platelet binding sequence (γ_A 400−411) (Kirschbaum et al., 1992). Heterodimeric peak 2 fibrinogen molecules contain one γ_A and one γ' chain. The variant γ' chain lacks the platelet binding sequence (Kirschbaum et al., 1992), is longer (427 residues), and has a more anionic, carboxyl-terminal sequence than the γ_A chain beyond position 408 (Wolfenstein-Todel & Mosesson, 1981). Plasma factor XIII copurifying with fibrinogen coelutes with peak 2 fibrinogen when separated on DEAE-cellulose (Mosesson & Finlayson, 1963a), and the factor XIII activity in the peak 2 fraction has, to date, not been easy to remove by any simple precipitation or chromatographic procedure. These observations raised the question as to whether there might be a specific interaction between the γ' chains in peak 2 fibrinogen and plasma factor XIII. This possibility prompted the investigation reported here.

MATERIALS AND METHODS

Tris[1] and glycine were obtained from Aldrich Chemical Co., Milwaukee, WI. PMSF and Coomassie Brilliant Blue R250 were purchased from Sigma Chemical Co., St. Louis, MO. Trasylol (aprotinin) was obtained from Miles Inc., Kankakee, IL. DEAE-cellulose (DE-52) was from Whatman Inc., Clifton, NJ. Human α-thrombin was a gift from Dr. J. Fenton, II (Division of Laboratories and Research, New York State Department of Health, New York).

Normal human fibrinogen fraction I-2 was isolated from pooled human citrated plasma by glycine precipitation (Kazal et al., 1963) and further purified as described by Mosesson and Sherry (1966). Fraction I-2 fibrinogen was subfractionated into peak 1 and peak 2 fibrinogen by chromatography on DE-52 (Finlayson & Mosesson, 1963; Mosesson et al., 1972) employing a concave gradient from 0.039 M Tris, 0.005 M H_3PO_4, 0.5 mM PMSF, 5 KIU/mL Trasylol, pH 8.55 (solution A), to 0.5 M Tris, 0.5 M H_3PO_4, 0.5 mM PMSF, 5 KIU/mL Trasylol, pH 4.2 (solution B). Gradient development was controlled by an FPLC (Pharmacia, Piscataway, NJ), programmed as detailed in Table 1. All buffer preparations and chromatography were performed at room temperature. For small protein samples ($\leq$10 mg), a 0.9 × 15 cm column with a 290 mL total gradient volume was employed. For larger protein samples (10−50 mg), a 0.9 × 30 cm column with a 580 mL gradient volume was used.

Table 1: FPLC Program for the Concave Elution Gradient[a]

% solution B	% total gradient volume	% solution B	% total gradient volume
5	23.6	50	89.1
10	50.7	60	92.1
15	62.6	70	94.7
20	71.0	80	96.9
25	76.2	90	98.8
30	81.4	100	100
40	85.7		

[a] After sample loading, the column is washed with 2 column volumes of solution A, and the gradient is then applied. A total gradient volume of 580 mL is employed using a 0.9 × 30 cm column, whereas a gradient volume of 290 mL is used with a 0.9 × 15 cm column.

Factor XIII B chain-enriched peak 2 fibrinogen was prepared from peak 2 fibrinogen containing bound zymogen by precipitation with ammonium sulfate at 20% saturation (w/v) at 4 °C. Peak 2 fibrinogen free of factor XIII A or B subunits was recovered from the supernatant solution by raising the ammonium sulfate concentration from 20% to 33% saturation (w/v) at 4 °C. The content of factor XIII subunits in the various fibrinogen fractions was assessed by SDS−PAGE on 8−25% Phastgels (Pharmacia) under nonreducing conditions. All fibrinogen subfractions were greater than 98% clottable. Fibrinogen concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient ($A^{1\%}_{1cm}$) of 15.1 (Mosesson & Finlayson, 1963b).

Plasma factor XIII was purified from pooled human citrated plasma (Lorand & Gotoh, 1970) and assayed as described by Loewy et al. (1961b). Factor XIII concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient ($A^{1\%}_{1cm}$) of 13.8 (Schwartz et al., 1973). The specific activity ranged from 2500 to 3000 units/mg. Sample purity and subunit composition were determined by SDS−PAGE on 9% polyacrylamide slab gels (Laemmli, 1970).

Placental factor XIII (A_2, Fibrogammin HS) was a generous gift from Dr. Hermann E. Karges (Behringwerke, Frankfurt, Germany). The A_2 subunits were separated from serum albumin in the preparation by differential precipitation with ammonium sulfate between 25% and 30% saturation (w/v). The precipitate was dialyzed against 50 mM citrate, 150 mM NaCl, pH 7.4, buffer containing 20% glycerol and stored at −80 °C. The specific activity was 2800 units/mg.

Ion-Exchange Chromatography of Fibrinogen/Factor XIII Mixtures. Fibrinogen (fraction I-2, peak 1, or peak 2 fibrinogen, 1−25 mg) was mixed with plasma factor XIII (0.05−5 mg) or placental factor XIII A_2 subunits (0.05−5 mg) and dialyzed against solution A (0.039 M Tris, 0.005 M H_3PO_4, pH 8.55). The sample was loaded onto a DE-52 column that had been equilibrated with the same buffer and then eluted with the gradient described above for separating peak 1 from peak 2 fibrinogen. Fractions of 1% of the total gradient volume were collected at a flow rate of 1 mL/min.

Fractions eluting in the regions corresponding to peak 1 fibrinogen, peak 2 fibrinogen, and factor XIII were dialyzed against 50 mM Tris, 100 mM NaCl, pH 7.4, and assayed for factor XIII activity (Loewy et al., 1961b). The elution positions of individual components were determined using the purified proteins.

Gel Sieving Chromatography of Fibrinogen/Factor XIII Mixtures. Mixtures of factor XIII and fibrinogen were dialyzed against 50 mM Tris, 100 mM NaCl, pH 7.4, and loaded onto a pair of Superose 6 columns (1 × 30 cm) connected in series to an FPLC. The columns were eluted with the above Tris buffer at a flow rate of 0.5 mL/min. Protein-containing fractions were assayed for factor XIII activity (Loewy et al., 1961b). Elution positions of the individual components were determined using the purified proteins.

The factor XIII binding properties of peak 1 and peak 2 fibrinogen in plasma were also tested by gel sieving chromatography. Fibrinogen was removed from plasma by heating to 56 °C for 3 min, rapidly cooling to 20 °C, and then centrifuging to remove thermally denatured fibrinogen. Plasma (5 mL) or heat-defibrinated plasma supplemented with peak 1 or peak 2 fibrinogen (3 mg/mL) was loaded onto a 1.5 × 90 cm column of Sepharose 6B-CL that had been equilibrated in 50 mM Tris, 100 mM NaCl, and 1 mM sodium citrate, pH 7.4. The column was eluted at a flow rate of 0.5 mL/min; 0.5 mL fractions were collected. Protein-containing fractions, as determined by the absorbance at 280 nm, were assayed for factor XIII activity (Loewy et al., 1961b).

RESULTS

When fraction I-2 fibrinogen was loaded onto a DEAE-cellulose column, two peaks of fibrinogen were resolved by the modified concave Tris−phosphate elution gradient (Figure 1). Peak 1 fibrinogen emerged from the column as a sharp peak centered at fraction 40. Peak 2 fibrinogen eluted much later as a smaller peak centered at fraction 85. Assaying these peaks for factor XIII activity revealed that all factor XIII activity eluted in the position of peak 2 fibrinogen (panel A). Purified plasma factor XIII alone (panel B) emerged as a single peak centered at fraction 66, positioned midway between peak 1 and peak 2 fibrinogen. A mixture of I-2 fibrinogen plus factor XIII resulted in two protein peaks eluting in the peak 1 and peak 2 fibrinogen positions, respectively (panel C). All of the factor XIII activity originally present in the I-2 fibrinogen sample as well as the added factor XIII activity was contained in the peak 2 fibrinogen fraction.

These results were extended using chromatographically isolated peak 1 or peak 2 fibrinogen. When factor XIII was applied to a small DEAE column and then eluted, a single protein peak emerged centered at fraction 72 (Figure 2, panel A). A mixture of peak 1 fibrinogen and factor XIII eluted from this system as two peaks: a major peak centered at fraction 40 and a smaller peak centered at fraction 72 (panel B). All factor XIII activity was contained in the second peak. An identical mixture of peak 2 fibrinogen and factor XIII emerged from this column as a single protein peak centered at fraction 90 (panel C). All factor XIII activity was associated with the single protein peak. The factor XIII

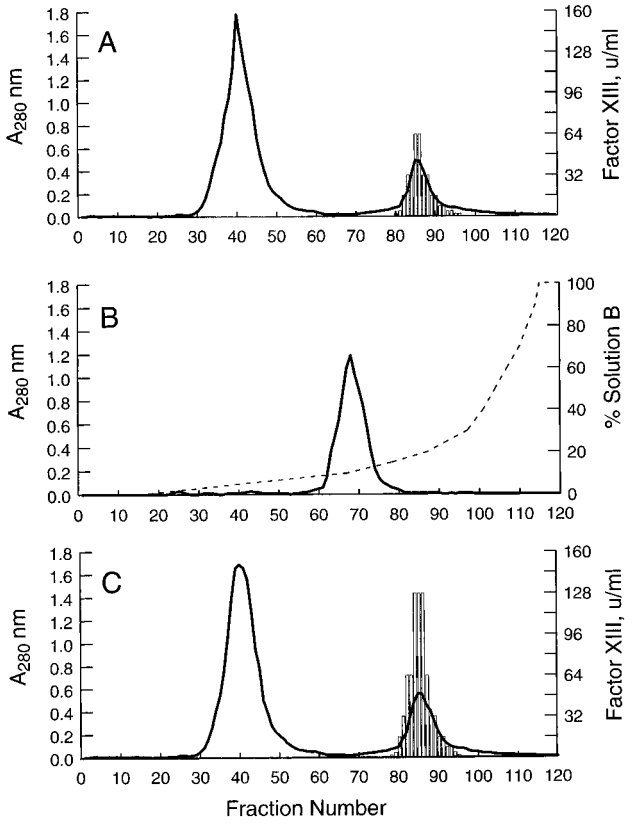

FIGURE 1: DEAE-cellulose chromatography of fraction I-2 fibrinogen without or with added plasma factor XIII. Samples were chromatographed on a DEAE-cellulose column (0.9 × 30 cm) using a concave Tris−phosphate elution gradient of 580 mL (dashed line in panel B). Panel A, fraction I-2 fibrinogen (25 mg); panel B, plasma factor XIII (10 mg); panel C, fraction I-2 fibrinogen (25 mg) supplemented with plasma factor XIII (2 mg). Protein-containing fractions were assayed for factor XIII activity (bars in panels A and C).

activity peak had been shifted away from its usual position into the more anionic position associated with peak 2 fibrinogen.

To determine whether the interaction between plasma factor XIII and peak 2 fibrinogen is mediated via the zymogen A subunit or B subunit, chromatographic experiments were repeated using placental factor XIII (A_2) that had been mixed with peak 2 fibrinogen free of B chains, or enriched in factor XIII B subunits (Figure 3). Placental factor XIII by itself (200 μg), on the DEAE column, eluted as a single protein peak centered at fraction 100 (results not shown). Its elution position was significantly later than that of plasma factor XIII and somewhat later than the peak 2−plasma factor XIII complex. Chromatography of a mixture of peak 1 fibrinogen with placental factor XIII (panel A), or B subunit-free peak 2 fibrinogen with placental factor XIII (panel B), resulted in factor XIII activity profiles that were identical to that of placental factor XIII alone. Mixtures of B subunit-enriched peak 2 fibrinogen with placental factor XIII resulted in a reproducible shift in factor XIII activity from the position of placental factor XIII to the position of the plasma factor XIII−peak 2 complex (panel C). This result strongly suggests that the interaction between plasma factor XIII and peak 2 fibrinogen occurs via the B subunit of plasma factor XIII.

The interaction between plasma factor XIII and peak 2 fibrinogen was also examined by gel sieving chroma-

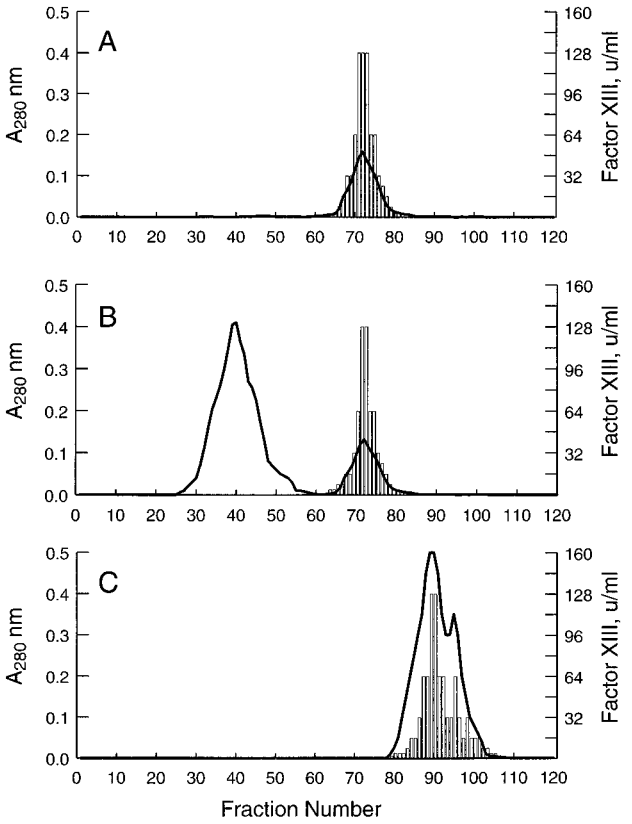

FIGURE 2: DEAE-cellulose chromatography of plasma factor XIII mixed with peak 1 or peak 2 fibrinogen. Samples were applied to a 0.9 × 15 cm column and eluted using the same gradient as in Figure 1 at a lower total gradient volume (290 mL). Panel A, plasma factor XIII alone (1.5 mg); panel B, peak 1 fibrinogen (5 mg) plus factor XIII (1.5 mg); panel C, peak 2 fibrinogen (5 mg) plus factor XIII (1.5 mg). Protein-containing fractions were assayed for factor XIII activity (bars).

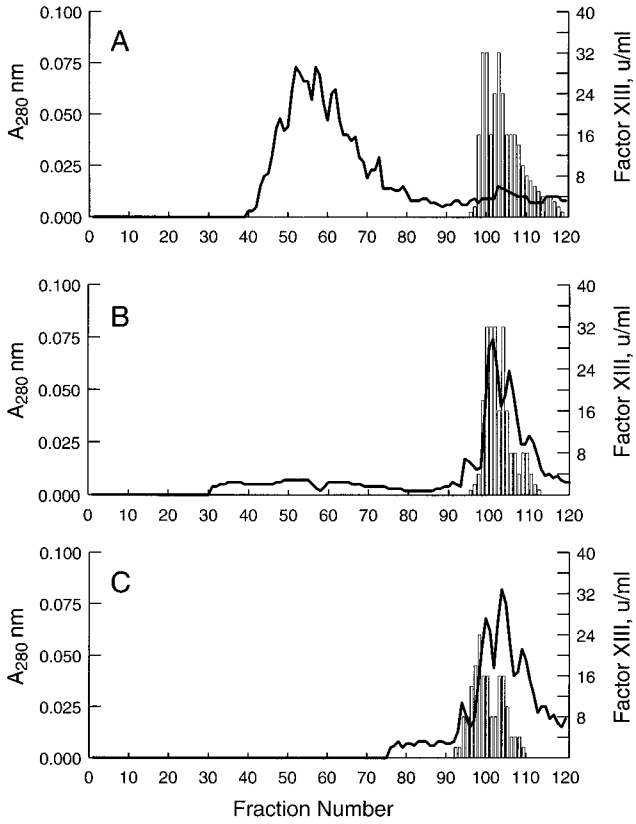

FIGURE 3: DEAE-cellulose chromatography of mixtures of placental factor XIII (200 μg) with 2 mg of peak 1 fibrinogen (panel A), 2 mg of factor XIII-free peak 2 fibrinogen (panel B), or 2 mg of factor XIII B chain-enriched peak 2 fibrinogen (panel C) using the same gradient as in Figure 2. Fractions were assayed for factor XIII activity (bars).

tography on Superose 6 (Figure 4). In this system, fibrinogen eluted as a single peak centered at fraction 35, and plasma factor XIII eluted at fraction 47 (panel A). A mixture of peak 1 fibrinogen and plasma factor XIII eluted as two peaks, with the majority of the factor XIII activity eluting in the second peak. A small amount of enzyme activity eluted with the trailing end of the peak 1 fibrinogen peak (panel B). Similar mixtures of peak 2 fibrinogen and plasma factor XIII eluted as a single peak with all of the factor XIII activity associated with the leading edge of the protein peak (panel C).

The same results were obtained when the elution profile of factor XIII activity was measured in plasma separated by gel sieving chromatography (Figure 5). Factor XIII activity in citrated human plasma emerged in a peak that was slightly ahead of the position of fibrinogen (panel A). Removing the fibrinogen by heat denaturation caused the factor XIII activity to elute from the column about five fractions later at an elution volume identical to that of purified factor XIII (panel B). Adding a physiological amount of peak 1 fibrinogen to the defibrinated plasma resulted in a minor change in the elution position. The majority of the enzyme activity eluted in the factor XIII position, with about 10% emerging earlier in the position of fibrinogen (panel C). Adding an equal amount of peak 2 fibrinogen shifted the elution profile such that the factor XIII activity emerged in the fibrinogen position (panel D).

DISCUSSION

Previous studies have shown that plasma factor XIII (A_2B_2) copurifies with fibrinogen and also coelutes with peak 2 fibrinogen when separated from peak 1 by DEAE chromatography (Mosesson & Finlayson, 1963a). Since the original gradient that was employed to separate peak 1 fibrinogen from peak 2 (Finlayson & Mosesson, 1963; Mosesson et al., 1972) did not result in base line separation between the two protein peaks, it was difficult to ascertain whether factor XIII was specifically bound to peak 2 fibrinogen or simply emerged independently at or near the peak 2 position. By refining the concave elution gradient, base line separation of peak 1 and peak 2 fibrinogen was obtained, and uncomplexed plasma factor XIII was resolved from factor XIII bound to peak 2 fibrinogen. The results from the DEAE-cellulose chromatography, coupled with those obtained from the gel sieving system, indicate that peak 2 fibrinogen, not peak 1, binds specifically to factor XIII.

Peak 1 fibrinogen contains two γ_A chains, whereas peak 2 molecules each contain one γ_A and one γ' chain (Wolfenstein-Todel & Mosesson, 1981). Previous studies on isolated γ' chains have indicated that they do not support platelet aggregation as do γ_A chains (Harfenist et al., 1984; Peerschke et al., 1986; Amrani et al., 1988; Kirschbaum et al., 1992). To date no function for this normally occurring variant γ chain sequence has been identified. From these present results, we can conclude that fibrinogen γ' chains function in plasma as carriers for factor XIII, evidently serving to

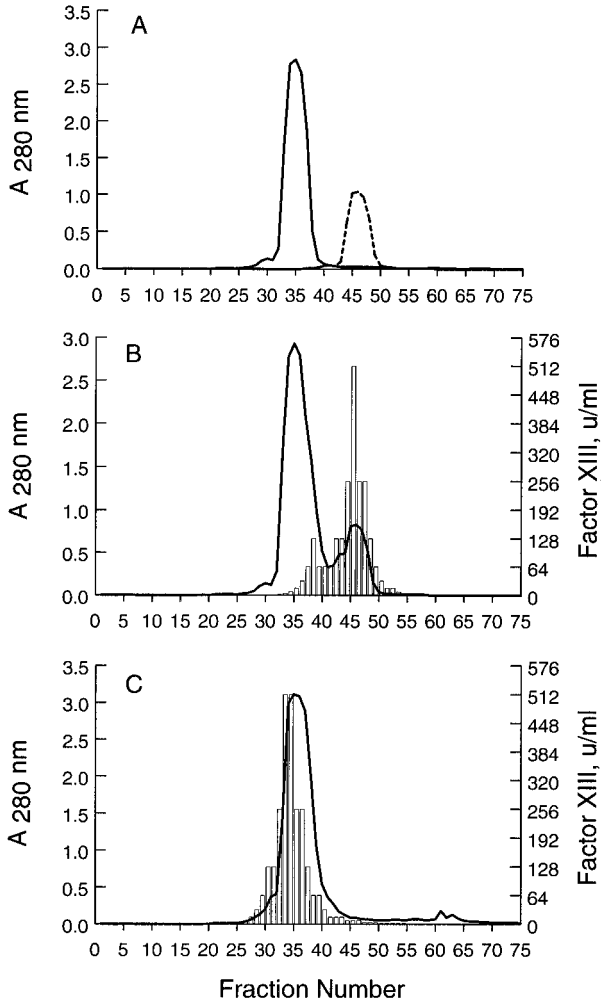

FIGURE 4: Gel sieving chromatography of peak 1 or peak 2 fibrinogen with plasma factor XIII. Fibrinogen (25 mg), plasma factor XIII (5 mg), or mixtures thereof were chromatographed on a pair of 1 × 30 cm Superose 6 columns attached in tandem to an FPLC. Panel A, peak 1 fibrinogen alone (solid line); factor XIII alone (dashed line); panel B, peak 1 fibrinogen plus factor XIII; panel C, peak 2 fibrinogen plus factor XIII. Protein-containing fractions from the fibrinogen−factor XIII mixtures were assayed for factor XIII activity (bars in panels B and C).

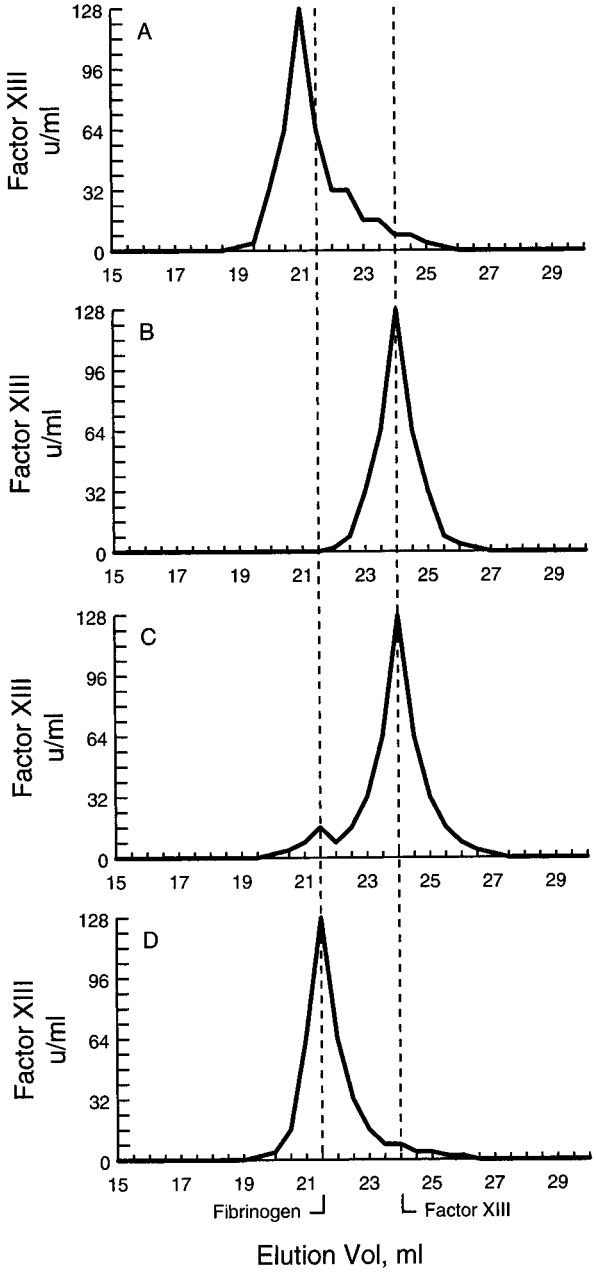

FIGURE 5: Gel sieving chromatography of heat-defibrinated plasma supplemented with peak 1 or peak 2 fibrinogen. Whole human plasma (5 mL) (panel A) or heat-defibrinated plasma (panel B) was chromatographed on a 1.5 × 90 cm column of Sepharose 6B-CL. In panel C, peak 1 fibrinogen was added at a final concentration of 3 mg/mL to heat-defibrinated plasma before chromatography. Panel D contained an equivalent amount of added peak 2 fibrinogen. The elution position of the factor XIII activity in these samples was determined by assaying all protein-containing fractions. Purified fibrinogen and factor XIII eluted at the positions indicated by the dashed lines.

deliver the zymogen to its site of action *in vivo*, i.e., the vicinity of the fibrin clot.

The apparent discrepant results with regard to factor XIII binding to fibrinogen can now be rationalized. When whole plasma (Loewy et al., 1961a; Loewy, 1972) or unfractionated fibrinogen preparations (Bannerjee & Mosesson, 1975) were used in binding studies, investigators observed binding of plasma factor XIII to fibrinogen. Binding was noted in these studies because the necessary components were present, i.e., peak 2 fibrinogen and plasma factor XIII. Studies that failed to demonstrate plasma factor XIII binding to fibrinogen were lacking one of these two essential components. For example, no evidence was found for complex formation between plasma factor XIII and fibrinogen by Chung (1972). The factor XIII-free fibrinogen employed in that study was evidently peak 1 fibrinogen, which lacks γ' chains. Several studies have suggested that factor XIII binds to fibrinogen by its A_2 subunit, a finding contrary to that reported here. These conclusions were based on binding studies of factor XIII A_2 subunits to surface-bound fibrinogen (Greenberg & Shuman, 1982) or reduced fibrinogen chains (Mary et al., 1987). These conditions may have exposed otherwise cryptic binding sites on the fibrinogen molecule or subunit chains.

The plasma factor XIII−fibrinogen interaction appears to be mediated by factor XIII B chains. Placental factor XIII, which is composed of only A chains, did not bind to peak 1 fibrinogen or to peak 2 fibrinogen that had been rendered free of factor XIII B chains. It did bind, however, to peak 2 fibrinogen that was enriched in B chains. The ratio of B chains to A chains in the factor XIII that copurifies with peak 2 fibrinogen is greater than 1, further suggesting that B chains alone bind to peak 2 fibrinogen. However, when

isolated B chains were added to peak 2 fibrinogen, the mixture rapidly came out of solution (unpublished observations), suggesting that A subunits may be needed either to stabilize B subunits or to stabilize the interaction between B subunits and peak 2 fibrinogen. Additional studies are required to address this question. Nevertheless, we can conclude that peak 2 fibrinogen γ' chains have a physiologically significant affinity for the B subunits of plasma factor XIII and that through this interaction fibrinogen serves as a carrier for the plasma zymogen in circulating blood.

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