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Human plasma fibrinogen heterogeneity: Evidence for an extended carboxyl-terminal sequence in a normal γ chain variant (γ′)

(carboxyl terminus/crosslinking/dansylcadaverine incorporation/fibrin)

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ABSTRACT **Two types of normal human plasma fibrinogen—peak 1 and peak 2—are distinguishable by DEAE-cellulose gradient elution chromatography. The elution characteristics of peak 2 fibrinogen, which amounts to about 15% of the total, are attributable to the presence of a γ chain variant, γ′, which is more negatively charged than γ chains and makes up about half of all such chains in that peak [Mosesson M. W., Finlayson, J. S. & Umfleet, R. A. (1972) *J. Biol. Chem.* 247, 5223–5227]. Analyses of reduced *S*-carboxymethylated fibrin that had first been incubated in the presence of Factor XIIIa plus the fluorescent amine donor dansylcadaverine (DNScad) showed that the same amount of this compound could be incorporated covalently into either type of γ chain. Furthermore, the DNScad-labeled COOH-terminal CNBr fragment (CNBr e) derived from the *S*-carboxymethylated γ chain was smaller than the DNScad-labeled fragment (CNBr e′) from the γ′ chain (M_r, 3200 and 4900) by about the same amount as the difference in size between the respective parent chains (M_r, 49,400 and 51,500). DNScad-CNBr e or DNScad-CNBr e′ could be further cleaved by trypsin to yield a smaller fluorescent fragment corresponding to the penultimate tryptic γ chain peptide containing the DNScad-glutamine acceptor and lysine donor crosslinking functions. The COOH-terminal amino acids of γ and γ′ chains were valine and leucine, respectively. The rates of Factor XIIIa-catalyzed crosslinking of peak 1 and peak 2 fibrin were the same, but peak 1 fibrin γ chains formed only one species of crosslinked dimer (γγ) whereas peak 2 fibrin γ chains yielded three (γγ,γγ′,γ′γ′). We conclude that γ′ chains are functionally normal but have an extended COOH-terminal sequence accounting for their more negative charge and larger size relative to γ chains.**

Human fibrinogen can be resolved by DEAE-cellulose gradient elution chromatography into two major fibrinogen peaks—peak 1 and peak 2 (1). Peak 2 fibrinogen amounts to about 15% of the total (1, 2) and differs from peak 1 fibrinogen by the presence of a unique class of γ chain, termed γ′ (3). The more negatively charged γ′ chains are distinguishable from other γ chains by gel electrophoresis at pH 8.6 or 2.7 (3), by isoelectric focusing (4), or by ion exchange chromatography (4). They account for approximately half of all such chains in the peak 2 fraction (3), suggesting that the general formula for molecules found in that peak is $(A\alpha)_2\,(B\beta)_2\,\gamma\gamma'$. A similar heterogeneity has been found for several other animal fibrinogens (5, 6); in the case of bovine fibrinogen, molecular species containing two γ′ chains have been demonstrated in a third, even later-eluting, chromatographic peak (6). A third fibrinogen peak has also been observed in human specimens (1, 2) but has not yet been examined in detail.

Studies by Martinez (7) indicate that human peak 1 and peak 2 fibrinogens are cleared from the circulation at the same rate and that neither type is converted to the other in the circulation. There are no significant differences in their sialic acid or phosphorus contents (1, 8). Furthermore, removal of sialic acid does not alter the behavior of the preparation with respect to the peak 1/peak 2 elution profile (4). In this regard, another type of γ chain heterogeneity, termed γ-1/γ-2 (3, 9), is superimposed upon the γ/γ′ heterogeneity. Compelling evidence has been presented that this heterogeneity is due to a difference in sialic acid content (10).

In the present investigation we localized and characterized the region accounting for the charge difference between γ and γ′ chains.

MATERIALS AND METHODS

Plasma fibrinogen fraction I-4 (11) (thrombin clottability, >95%), was prepared as described (12). Fractions enriched in peak 1 and peak 2 fibrinogens were prepared from this material by gradient elution chromatography on DEAE-cellulose (Whatman DE-23) (1–4). Factor XIII was prepared as described by Kazama *et al.* (13) up to the chromatography step. Its activity was 1280 Loewy units/ml (14).

Crosslinking of fibrin was carried out under the following conditions: fibrinogen, 0.5–3 mg/ml; cysteine·HCl (adjusted to pH 7.4 with Tris), 7–10 mM; Ca^{2+}, 15–25 mM; human thrombin (lot H-1, Bureau of Biologics, Food and Drug Administration), 1–2 USP units/ml. Components of the crosslinking mixture were diluted with 0.15 M NaCl/0.01 M Tris·HCl, pH 7.4. When desired, the crosslinking mixture was supplemented with Factor XIII at a final concentration of 3–13 Loewy units/ml. For certain experiments, crosslinking was carried out in the presence of 5 mM dansylcadaverine (DNScad; Sigma) to facilitate incorporation of this compound into glutamine acceptor sites in fibrin (15, 16).

When comparative studies of crosslinking rates were carried out, Factor XIII activity in the fibrinogen preparations was first inactivated in the presence of 3.3 M urea (17); the material was then subjected to dialysis against 0.3 M NaCl and supplemented with the desired amount of Factor XIII prior to crosslinking. Periodic sampling and inhibition of crosslinking for subsequent electrophoretic analysis was carried out by solubilization in 2% dithiothreitol/10 M urea/2% $NaDodSO_4$.

Reduction and *S*-carboxymethylation was performed as described (18). DEAE-Cellulose gradient elution chromatography of *S*-carboxymethyl (*S*-CM) derivatives was carried out in 8 M urea at pH 7.0 with a Tris phosphate gradient from 0.01 to 0.2 M (4). CNBr cleavage of *S*-CM peptides was carried out as described by Gross and Witkop (19).

Polyacrylamide gel electrophoresis was performed in the system described by Weber and Osborn (20) at 5–8 mA per

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Abbreviations: CM, carboxymethyl; DNScad, dansylcadaverine.

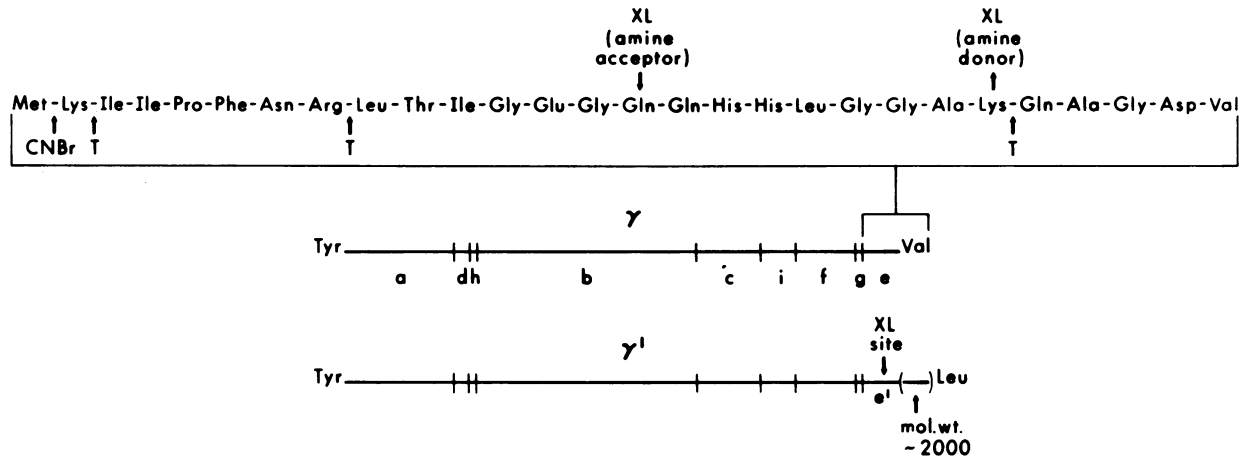

FIG. 1. (*Center* and *Bottom*) Diagram of the structure of γ and γ′ chains. CNBr cleavage points along the chain are indicated by vertical lines. The various CNBr peptides are designated by letters according to Henschen *et al.* (30). The reduced COOH-terminal CNBr peptide from the γ′ chain is designated e′. (*Top*) Amino acid sequence of CNBr e (29), with tryptic (T) and CNBr cleavage points and acceptor and donor crosslinking (XL) sites indicated.

tube, in gels containing 9–12% polyacrylamide. Slab gel electrophoresis was carried out in 10% polyacrylamide gels (9 × 15 × 0.15 cm) at 8 mA for 22 hr in the Laemmli system (21). Densitometric scanning of slab gels that had been stained with Coomassie brilliant blue was performed in an Aminco filter fluorometer equipped with a horizontal thin-layer scanning attachment.

NH_2-Terminal analysis was performed by an adaptation (22) of the DNS-Cl method of Gray and Hartley (23). COOH-Terminal analysis was performed by digestion with carboxypeptidase A (24). The protein to be analyzed was solubilized in 0.2 M *N*-ethylmorpholine acetate buffer at pH 8.0 to which $NaDodSO_4$ had been added to a final concentration of 50 mM; the mixture was then dialyzed against water to remove unbound detergent. After dialysis, *N*-ethylmorpholine buffer was again added to a final concentration of 25–50 mM and carboxypeptidase A digestion (phenylmethanesulfonyl fluoride-treated carboxypeptidase A, Millipore, Freehold, NJ) was carried out at room temperature overnight at an enzyme/substrate ratio of 1:100 (wt/wt). After digestion, the protein was precipitated with trichloroacetic acid at a final concentration of 5% (wt/vol), and the supernatant solution was applied directly to an amino acid analyzer (JEOL, Model JLC-6AH).

Peptide mapping experiments were carried out by a minor modification of the method of Katz *et al.* (25). Dansylated peptides were detected under UV light. To visualize other peptides, the paper was treated with fluorescamine (Fluram, Roche Diagnostics) (26).

RESULTS

As expected (27), the NH_2-terminal amino acid of both γ and γ′ chains was tyrosine. Overnight incubation of γ chains with carboxypeptidase A (five analyses) yielded the following mean values (residue/5 × 10^4 g): valine, 0.72; aspartate, 0.27; glycine, 0.25; alanine, 0.21; glutamine,* 0.21. These findings are consistent with the known COOH-terminal sequence of this chain (28, 29) (Fig. 1). In contrast, each of three γ′ chain preparations yielded only leucine; the mean value was 0.52 residue/5 × 10^4 g.

Most earlier assessments of the migration rates of γ and γ′ chains in $NaDodSO_4$ gels (20) had been conducted on reduced preparations of chromatographic fibrinogen fractions (3) and had given little reason to suspect that significant size differences might exist. Under our usual conditions of electrophoresis (Fig. 2, gels 1 and 2) both peak 1 and peak 2 fibrinogen γ chains migrated as single bands. However, close inspection indicated that the peak 2 γ chain position band usually was somewhat broader than that of peak 1. By varying the conditions of electrophoresis (e.g., 10% acrylamide, 4 mA per gel, 16 hr; see gels 9 and 10), the relative broadness of the peak 2 γ chain band could be more easily appreciated. Occasionally, it even could be resolved into two bands, although sharp definition was never obtained.

* Our chromatographic system does not distinguish glutamine from asparagine and threonine but, because the COOH-terminal γ chain sequence is known to contain glutamine (28), we have assumed its identity.

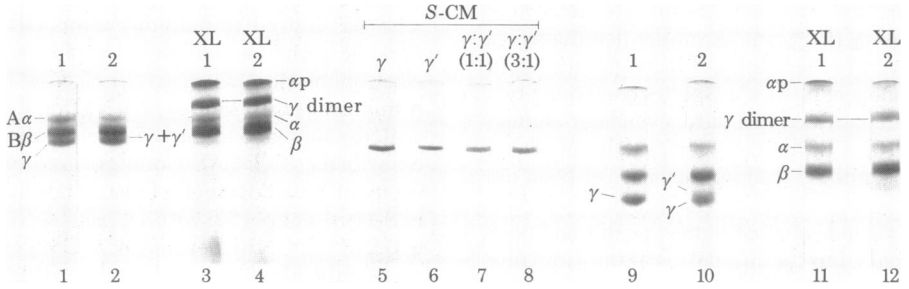

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis (20) under various running conditions. Electrophoresis was carried out in 9% polyacrylamide gels at 6 mA per tube for 5 hr (gels 1–4) or for 5.5 hr (gels 5–8) or in 10% polyacrylamide gels at 4 mA per tube for 16 hr (gels 9–12). Samples in gels 1–4 and 9–12 had been reduced with dithiothreitol, whereas those in gels 5–8 were reduced *S*-CM derivatives. All gels were stained with Coomassie brilliant blue. XL, Crosslinked; α_p, α polymer; 1 and 2, fibrin peaks 1 and 2, respectively.

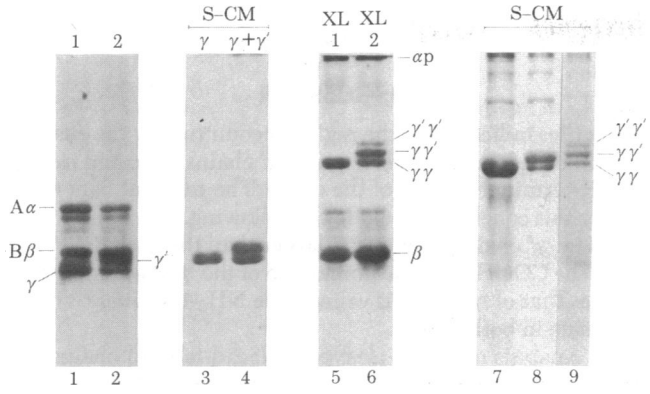

FIG. 3. NaDodSO$_4$/polyacrylamide slab gel electrophoresis (10% polyacrylamide) in the Laemmli system (21). Electrophoresis was carried out at 8 mA for 21 hr. Lanes 1, 2, 5, and 6 contained material that had been reduced with dithiothreitol; lanes 3, 4, 7, 8, and 9 contained reduced *S*-CM derivatives. Lanes 1–6 were from one electrophoretic experiment, and lanes 7–9 were from another. Lanes 7, 8, and 9 represent successive pooled chromatographic fractions from the γ dimer peak of crosslinked, reduced, *S*-CM fibrin (fraction I-4). The gels were stained with Coomassie brilliant blue. For abbreviations, see legend to Fig. 2.

Electrophoresis of isolated *S*-CM γ and γ′ chains under standard conditions at optimal protein load provided an explanation for this observation. The results (Fig. 2, gels 5–8) showed that γ′ chains migrated slightly less anodally than did γ chains. Their relative positions in this system corresponded to a M_r difference of about 2000 [49,400 (22) vs. 51,500].

In the Laemmli system (21) the apparent M_r of the γ′ chain, based upon the migration rates of α, β, and γ chains, was 52,000–53,000 (Fig. 3), and the γ′ chain could be readily appreciated as a discrete band. In this system, *S*-CM γ and γ′ chains behaved anomalously in that they both migrated significantly less anodally than their reduced counterparts, yielding apparent M_r values that are unrealistically high.

In order to compare functional aspects of γ and γ′ chains, fraction I-4 fibrin was crosslinked in the presence of DNScad, a fluorescent amine that serves as a pseudo amine donor in the presence of Factor XIIIa (15) and is thereby incorporated into glutamine acceptor sites on γ chains (28, 31) and α chains (31).

The DNScad-labeled fibrin was reduced, *S*-carboxymethylated, and subjected to gradient elution chromatography on DEAE-cellulose in the presence of urea (Fig. 4). The elution profile, as assessed by absorbance at 280 nm, showed the presence of both the γ and γ′ chain peaks in relative amounts expected for material derived from fraction I-4 fibrinogen [i.e., ≈7–8% γ′ chains (3, 4)]. The isolated DNScad-labeled chains showed the same relative difference in electrophoretic migration as had been observed for the *S*-CM chains themselves. The relative fluorescence intensity per mg protein was the same for each chromatographic peak, thus indicating that γ′ chains, like γ chains (28), contain a single glutamine acceptor site.

The DNScad-labeled *S*-CM γ and γ′ chains were digested with CNBr and the resulting fragments were analyzed by electrophoresis (Fig. 5). The fluorescent COOH-terminal CNBr fragment from the γ chain [CNBr e (30)] occupied a more anodal position than did the corresponding fragment from the γ′ chain (CNBr e′). The M_r of DNScad-CNBr e′ was estimated to be 4900 compared with the computed value of 3200 for DNScad-CNBr e. Counterstaining of the same gels with Coomassie brilliant blue showed several bands corresponding to both nonfluorescent and fluorescent CNBr fragments. There were no differences in these staining patterns other than those attributable to the anodal positions occupied by DNScad-CNBr e and DNScad-CNBr e′, respectively.

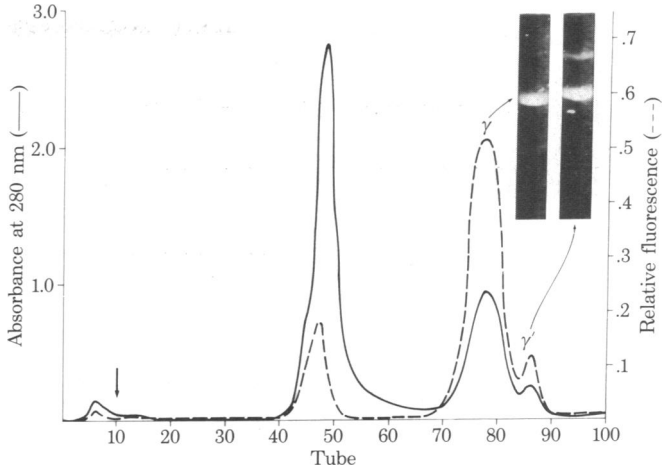

FIG. 4. DEAE-Cellulose gradient elution chromatography in 8 M urea of 400 mg of reduced, *S*-CM, DNScad-labeled fibrin (fraction I-4) on a 2.5 × 40 cm column. The vertical arrow indicates the start of the pH 7 Tris phosphate gradient [0.01 to 0.2 M phosphate (4)]. Each of the nine chambers of the gradient was 150 ml; fractions were 15 ± 0.4 ml. Absorbance at 280 nm (—) and relative fluorescence (---; excitation wavelength, 355 nm; emission wavelength, 525 nm) are indicated. The fluorescent band pattern obtained by NaDodSO$_4$ gel electrophoresis (20) (9% polyacrylamide) is shown for the *S*-CM γ and γ′ peaks. A small amount of fluorescent γ dimer was also eluted in the γ′ peak.

To evaluate the possible presence of the lysine crosslinking donor site or other lysine or arginine residues in DNScad-CNBr e′, we subjected the CNBr digests to tryptic hydrolysis. NaDodSO$_4$ gel electrophoresis (12% polyacrylamide) (20) showed that the respective DNScad-labeled CNBr fragments had been further cleaved (data not shown). In both cases, the fluorescent tryptic bands migrated in the same position, suggesting that tryptic cleavage of the CNBr fragments had occurred in the same place.

We also compared the tryptic digests by peptide mapping (Fig. 6). Each map of DNScad-labeled γ or γ′ chains or a 1:1 mixture of these yielded a single major fluorescent DNScad spot in the same position, thus indicating that the two fragments were identical. Fluorescamine counterstaining of these maps showed many spots, and no clear differences could be discerned among them (data not shown).

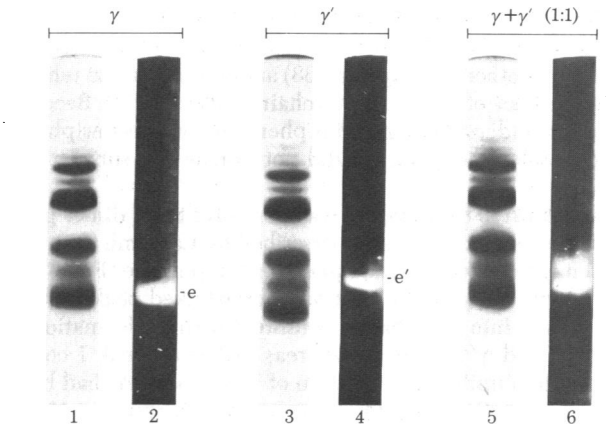

FIG. 5. NaDodSO$_4$ gel electrophoresis (20) (12% polyacrylamide) of CNBr-digested DNScad-labeled, *S*-CM γ and γ′ chains. Electrophoresis was carried out at 6 mA per gel for 4 hr. For estimation of the M_r of DNScad-labeled CNBr e′ (indicated by e′) we used the DNScad-labeled CNBr e position (indicated by e) [computed M_r 3200 (29)] and iniprol [M_r 6500 (32)] as markers. Gels 1, 3, and 5 were stained with Coomassie brilliant blue after they had been photographed under UV light (gels 2, 4, and 6). The positions of DNScad-labeled CNBr e and CNBr e′ in the fluorescent gels were also marked by a razor nick prior to staining.

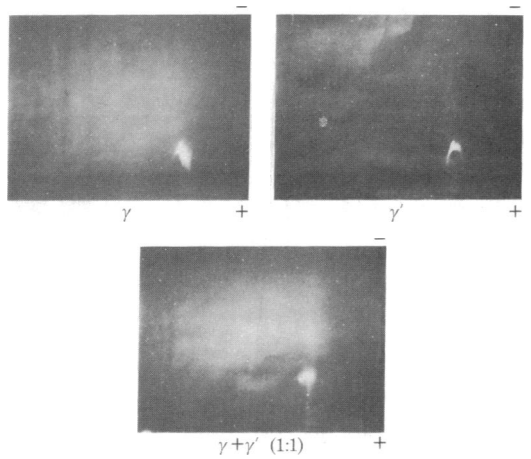

FIG. 6. Tryptic peptide maps of DNScad-labeled *S*-CM γ and γ' chains that had been digested with CNBr. The electrophoretic direction is vertical, with anode toward the bottom; the chromatographic direction is horizontal. The maps were photographed under UV light.

Theoretically, if crosslinking between γ and γ' chains takes place in a nonselective manner, then three types of γ dimers should be formed when both γ and γ' chains are present in a crosslinking mixture (i.e., γγ, γγ', and γ'γ'), and they should be distinguishable from one another on the basis of both size and charge. Furthermore, considering that the γ chain crosslinking site is situated near the COOH terminus (Fig. 1) and that the essential difference between γ and γ' chains involves COOH-terminal sequences, it seemed possible that this difference could have an effect on the rate of γ dimerization. In order to assess these possibilities, the following experiments were carried out.

Fibrin was formed, in the presence of Ca^{2+}, from urea-treated peak 1 and peak 2 fibrinogens that had subsequently been supplemented with Factor XIII (3 Loewy units/ml, final concentration). No significant differences were observed with regard to the rate of γ dimer formation (results not shown).

Analyses of reduced, crosslinked fibrin specimens in the Weber and Osborn system gave little indication of the presence of more than one type of dimerized γ chain in either specimen (Fig. 2, gels 3, 4, 11, and 12). The apparent M_r of material occupying this position (82,000–86,000) was lower than the sum of the constituent γ chains, an unusual behavior that has been observed by other investigators (33) and which may be related to the presence of covalent interchain crosslinks (34). Because a detailed understanding of this phenomenon was peripheral to the problem at hand, we did not pursue this subject further.

In an effort to find a better way to resolve the γ dimer position, we evaluated the system described by Laemmli (21), and found it to be more than adequate for this purpose (Fig. 3). In this system, the γ dimer position of crosslinked peak 2 fibrin was resolved into three bands, consistent with the formation of γγ, γγ', and γ'γ' dimers, whereas that from peak 1 corresponded to a uniform population of γγ dimers. As had been the case in the Weber and Osborn system, the apparent M_rs of the various dimers (84,500, 88,000, and 91,000) were somewhat lower than the values expected.

Further evidence supporting the identity of the various γ dimer bands was obtained by electrophoretic analysis of sequentially pooled fractions from the γ dimer peak resulting from chromatography of *S*-CM crosslinked fibrin (Fig. 3, lanes 7–9). The elution sequence of the dimerized chains (γγ, γγ', γ'γ') corresponded to their relative negative charge distribution. Densitometric scanning of the γ dimer bands from peak 2 fibrin indicated that the three species were present at a relative ratio (anodal to cathodal) of 35:45:20 (see below).

DISCUSSION

Our studies indicate that the region accounting for the charge and size differences between γ and γ' chains is located in the COOH-terminal portion of the chain. The relevant data supporting this conclusion include the following.

(*i*) The γ' chain is somewhat larger than the γ chain.

(*ii*) The COOH-terminal amino acid of the γ' chain is leucine whereas that of γ chains is valine. The NH_2-terminal residue is tyrosine in both cases.

(*iii*) Analysis of CNBr-cleaved DNScad-labeled chains indicated that the fluorescent COOH-terminal peptide derived from the γ' chain was larger than the fluorescent γ chain peptide by about the same amount as the difference between the two intact chains (i.e., 2000). As assessed by staining with Coomassie brilliant blue after $NaDodSO_4$ electrophoresis, other CNBr γ and γ' chain peptides were the same.

(*iv*) In each case, tryptic hydrolysis of the fluorescent CNBr digests yielded a smaller fluorescent peptide containing the DNScad-labeled glutamine acceptor crosslinking site. These DNScad peptides did not differ from one another when compared by $NaDodSO_4$ gel electrophoresis and by peptide mapping. This suggests that tryptic cleavage results in formation of identical fluorescently labeled fragments corresponding to the penultimate tryptic γ chain peptide. Taken together with other data, this finding indicates that the differences between γ and γ' chains are located in the sequence distal to this peptide.

Failure to recognize the small difference in size between γ and γ' chains (3) stems from the fact that, under standard conditions of $NaDodSO_4$ gel electrophoresis (Fig. 2), the minor size differences are usually not well resolved.

Francis *et al.* (35) used a gradient gel electrophoresis system similar to the Laemmli system and identified a minor population of γ chains (termed "γ_B") which are somewhat larger than the major population of γ chains ("γ_A"). It seems evident that they were dealing with the same heterogeneity as we have characterized here.

It can be inferred from previous studies that both γ and γ' chains are capable of crosslinking (3). Our data extend those results in providing evidence that γ' chains are functionally normal and crosslink nonselectively with one another. First, analyses of DNScad-labeled *S*-CM γ and γ' chains and their tryptic fragments indicated that the glutamine acceptor site and the lysine donor site, respectively, were the same in both types of chains.

Second, as assessed from periodic sampling of peak 1 and peak 2 fibrin crosslinking mixtures, the relative rates of utilization of these sites by γ and γ' chains did not differ. Finally, electrophoretic analysis of crosslinked peak 1 and peak 2 fibrin specimens and chromatographic fractions containing *S*-CM derivatives of γ dimers indicated that dimers formed from peak 2 fibrin were of three types (γγ, γγ', and γ'γ'), whereas those from peak 1 fibrin formed only γγ dimers.

The relative amount of each type of γ dimer that formed from peak 2 fibrin was very close to that predicted for nonselective dimerization of these chains—that is, nonselective dimerization of γ chains in a fibrin preparation having a γ/γ' ratio of 57:43 [i.e., peak 2 fibrinogen (3)] should produce γγ, γγ', and γ'γ' dimers in the ratio 33:49:18. This theoretical ratio, which was calculated from the binomial equation, was close to that which we actually found by densitometric scanning—namely, 35:45:20.

Our findings on this last aspect of γ chain crosslinking differ from those of Francis *et al.* (35) who concluded from their re-

sults that γ_A and γ_B (i.e., γ and γ') chains dimerize selectively and only with chains of the same type (i.e., $\gamma_A\gamma_A$ and $\gamma_B\gamma_B$). We believe that this disparity is related to the fact that their fibrinogen preparation had not been subfractionated chromatographically. Thus, its γ' chain population amounted to only about 7% of the total (3, 4) (Fig. 4). The predicted ratio of dimers $\gamma\gamma/\gamma\gamma'/\gamma'\gamma'$ forming from such fibrinogen is 86.5:13:0.5. The first two numbers, representing the $\gamma\gamma$ and $\gamma\gamma'$ components, are close to the distribution found by Francis *et al.* for the two dimer bands that they observed (i.e., 84:16). A third band, reflecting the minor population of $\gamma'\gamma'$ dimers in their preparations, was probably too faint to have been visualized.

Our data indicate that γ' chains have an extended COOH-terminal sequence relative to γ chains but otherwise, they have the same sequence from NH_2-terminal tyrosine to at least as far as the lysine donor crosslinking site that is located in the COOH-terminal CNBr peptide, CNBr e (Fig. 1). We have no evidence yet as to whether the ultimate pentapeptide sequence of the γ chain is retained in γ' chains.

Analysis of mutant γ chains (γ Paris I) from a congenitally abnormal fibrinogen molecule (fibrinogen Paris I) showed that these abnormal chains expressed the γ/γ' heterogeneity (4). This observation is consistent with the differences between γ and γ' chains being due to modification of a single gene product at the level of translation.

There are at least two peptide processing events that are consistent with the above postulation and that can explain our findings. One possibility is that the γ' chain is a naturally occurring "readthrough" protein. Such proteins are produced when a termination codon is recognized by a type of tRNA termed suppressor tRNA, which consequently inserts an amino acid (36), a process which results in continuing addition of amino acids to the peptide chain until the next termination codon is reached and properly read. Such readthrough proteins have been best studied in viral systems, such as in $Q\beta$ infection of *Escherichia coli* (37–39). Naturally occurring readthrough in a mammalian system has also been described by Geller and Rich (40).

An alternative possibility is that γ' chains represent an incompletely processed gene product from which a COOH-terminal peptide can be cleaved to produce γ chains. Included among examples of such proteins are the precursors of collagen (41–43), glucagon (44–46), and vasopressin (47), which are synthesized with both NH_2- and COOH-terminal extensions. We favor this possibility because it is also consistent with recent studies of fibrinogen polypeptide chain synthesis in rat hepatocytes (48) in which newly translated peptides reportedly migrated slightly less anodally in $NaDodSO_4$-containing systems than did chains obtained from fibrinogen molecules. If γ chains do arise by cleavage of precursor γ' chains, the event must take place intracellularly because Martinez (7) has shown that there is no interconversion between peak 2 and peak 1 fibrinogen molecules in the circulation.

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