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The minor form α′ chain from lamprey fibrinogen is rapidly crosslinked during clotting

(terminal domain/Western blotting)

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ABSTRACT **Lampreys have two genes for the α chains of fibrinogen, the second of which encodes a minor form with a carboxyl-terminal domain homologous to the carboxyl-terminal domains of β and γ chains. Initially, we referred to the alternative chain as α-II; we now use the designation α′ in order to facilitate reference to crosslinked dimers. Antisera raised to synthetic peptides based on the cDNA sequence confirmed that the α′ chain was present in fibrinogen prepared directly from plasma. The same antibodies were used to determine the size and properties of the carboxyl-terminal domain after its release by mild tryptic digestion, a fragment of apparent molecular weight 35,000–40,000 being produced. Unlike fragment D generated in the same digestions, the α′ fragment did not bind to Gly-Pro-Arg or Gly-Val-Arg peptide affinity columns. During clotting under conditions where factor XIII is active, the α′ chains became crosslinked very much more rapidly than ordinary α chains, the principal product being an apparent dimer, but smaller amounts of higher multimers being detectable. The crosslinking was inhibited by various amines, as well as by peptides that prevent polymerization.**

Vertebrate fibrinogens are invariably composed of three pairs of nonidentical subunits $(\alpha\beta\gamma)_2$. Ordinarily, β and γ chains are homologous over their entire lengths, whereas α chains are related only over the course of their amino-terminal thirds. However, alternative α chains have been identified that have domains at their carboxyl termini resembling those of β and γ chains (1, 2). In the lamprey (1) the alternative chain is encoded by a separate gene, but in humans (2), and by analogy in chickens (3), the second form is the result of alternative splicing.

In either case, the abundance of the alternative form must be low in that it has gone undetected in thousands of studies on various fibrinogens over the last 30 years. In lampreys, the message abundance in liver is significantly lower than the ordinary α-chain message, and certainly <20% (1). Estimates of the levels in circulating plasma based on isolated fibrinopeptides A and A2 put the level of α′ chain at about 10% of that of the ordinary α chain (4).

In this paper we report the characterization of lamprey fibrinogen containing α′ chains by the use of antibodies generated against synthetic peptides patterned on sequences obtained from cDNA. Our initial goal was to confirm the existence of full-length α′ chains in fibrinogen isolated from lamprey plasma. Beyond that, we were interested in its general structural and functional features. A recent report characterizing the equivalent chains in human fibrinogen, noting that the extra domains seem always to occur two per molecule, postulated that they are centrally located and may be tied together by a disulfide bond (5). Accordingly, we undertook experiments to see whether such a situation might exist in lampreys. To this end, we used a limited trypsin treatment to generate the equivalent of fragments D and E from lamprey fibrinogen, anticipating that such a digestion would also release the α′ carboxyl domain more or less intact. We used these same digests to find whether the α′ carboxyl-terminal domain binds to Gly-Pro-Arg and Gly-Val-Arg peptide columns, those peptides constituting the amino termini of the α and β chains, respectively. It is generally accepted that the initial stage of fibrin polymerization involves an interaction between Gly-Pro-Arg "knobs" and "holes" located on the terminal domains included in fragment D. In lampreys, both the α and α′ chains have Gly-Pro-Arg amino termini after treatment by thrombin. Finally, we followed the course of the α′ chain during clotting under conditions where factor XIII was active. These latter studies provided key insights into the likely function of the alternative α chains.

MATERIALS AND METHODS

Lamprey fibrinogen was prepared from frozen lamprey plasma by a single precipitation step with cold 50% ethanol containing 55 mM sodium citrate buffer (pH 6.5). The precipitate was dissolved in one-fifth of the starting plasma volume of 0.3 M NaCl. SDS/PAGE under reducing conditions revealed three major bands corresponding to the α, β, and γ chains. Occasionally, these crude preparations were contaminated with plasma albumin, the major protein component of lamprey plasma. Lamprey albumin is a glycoprotein with a molecular weight of 175,000 (6).

Clotting was effected by the addition of thrombin in the presence or absence of 10 mM $CaCl_2$ and 10 mM cysteine, according to whether or not the activation of factor XIII was desired. A description of the lamprey thrombin used has appeared previously (7); bovine thrombin was obtained from Parke–Davis. Trypsin and soybean trypsin inhibitor for the limited proteolysis of lamprey fibrinogen were purchased from Sigma. Affinity chromatography of fragment D was conducted on Gly-Pro-Arg columns of the sort first described by Kuyas *et al.* (8); these were used under conditions reported previously (9). A similar column derivatized with Gly-Val-Arg (amino terminus of lamprey fibrin β chain) was also prepared and tested.

Synthetic peptides corresponding to three different portions of the α′ chain were prepared at the University of California at San Diego, Peptide Synthesis Facility. The sequences chosen were residues 95–103 (QVVTDREDT), 208–218 (EHARSLHPYSG), and 613–622 (FRRVQIPIVE). The original α′ residue numbering (1) has been revised to include the additional two residues identified as being part of the mature chain (4). We refer to these as regions A, B, and C, and the antisera raised against these peptides are called antiserum A, antiserum B, and antiserum C. In each case KG or KGG residues were added to the amino termini of the actual sequences as tethers for coupling to carriers by a glutaraldehyde procedure (10).

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Inadvertently, peptide B had two erroneous residues adjacent to the tether (EH instead of DD). The carboxyl-terminal octapeptide was correct, however, and antibodies were obtained that reacted strongly with the α' chain.

Antibodies were raised in rabbits against conjugates of peptide and bovine plasma albumin. Antisera were assayed by ELISAs on microtiter plates coated with ovalbumin–peptide conjugates. Plates were developed with an alkaline phosphatase assay employing *p*-nitrophenyl phosphate as a substrate. After suitable booster injections and the achievement of high titers, rabbits were exsanguinated and the serum was stored at −20°C.

Western blot analysis was conducted along the lines described by Towbin *et al.* (11). After SDS/PAGE, proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were then treated overnight with 5% dry milk (Carnation) in Tris-buffered saline (TBS: 20 mM Tris, pH 8.0/150 mM NaCl). They were then incubated with high dilutions of antisera (1:500 or 1:1000) for 3 hr at room temperature, washed three times with TBS/0.05% Tween 20, and then incubated with a 1:3000 dilution of a secondary goat anti-rabbit IgG–alkaline phosphatase conjugate (Bio-Rad). All dilutions were with 1% dry milk/TBS. Color development was attained by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma).

RESULTS

Lamprey fibrinogen prepared by a single-step precipitation direct from plasma contains measurable amounts of a fibrinogen species containing the α' chain as visualized by Western blotting. Antisera B and C at suitable dilutions were specific for the α' chains, but antiserum A crossreacted with the major α chain as well and was not used thereafter.

Under nonreducing conditions, Western blotting with antiserum C gave a single band in between the two major Coomassie blue-staining fibrinogen components, indicating that mixed molecules containing both ordinary α and α' components do not occur (Fig. 1). Under reducing conditions, the same antiserum revealed a single band moving only slightly faster than the major α-chain component. Indeed, once the position was determined by Western blotting, it was possible to visualize the corresponding band by Coomassie blue staining of overloaded gels (Fig. 1). The calculated molecular weight of the α' chain is 68,145 (amino acids only), in contrast with the molecular weight of 96,722 of the major α chain. That the

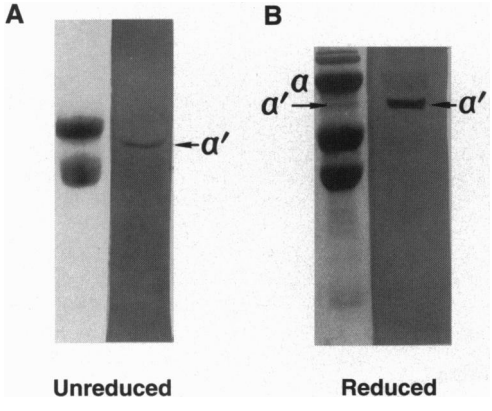

FIG. 1. Demonstration of a single lamprey fibrinogen species containing a minor form of α chain (α') by Western blotting with antiserum C (right lanes) and Coomassie blue staining (left lanes) of SDS/polyacrylamide gels. (*A*) Unreduced lamprey fibrinogen run on a 5% polyacrylamide maxigel (20 cm × 20 cm) for 20 hr at 50 V. (*B*) Reduced lamprey fibrinogen run on a 7.5% polyacrylamide minigel (6 cm × 8 cm) for 90 min at 50 V.

major α-chain component lacks carbohydrate (18) diminishes the difference, however, and accounts for why the alternative form, which has three putative asparaginyl glycosylation sites, is found to travel only slightly faster in polyacrylamide gels (Fig. 1). The ratio of major to minor forms by Coomassie blue staining appears to be somewhere between 10:1 and 20:1, consistent with earlier estimates based on measurements of the fibrinopeptides A (4).

Fragment Characterization. Fragments equivalent to D and E are released from fibrinogen by mild digestion with trypsin (18). Fortuitously, we had previously worked out a set of conditions that optimizes the yield of these fragments from human fibrinogen, and we were able to apply these directly to the lamprey fibrinogen. Lamprey fibrinogen (5 mg/ml) was incubated with trypsin (0.05 mg/ml) (100:1 weight ratio) for 4 hr at 22°C in 0.3 M NaCl/5 mM $CaCl_2$/50 mM imidazole, pH 7.0. The digestion was stopped by the addition of soybean trypsin inhibitor (0.15 mg/ml). Digests were then subjected to either gel filtration on Sephadex G-100 or affinity chromatography on Gly-Pro-Arg or Gly-Val-Arg peptide columns.

Estimated Size by Gel Filtration. Light tryptic digests of lamprey fibrinogen were passed over a Sephadex G-100 column (2.5 cm × 70 cm) that had been equilibrated with the same buffer as had been used in the digestion (Fig. 2). The eluate was fractionated, pools were concentrated by centrifugation in Centricon filters (molecular weight cutoff, 10,000; Amicon), and aliquots were subjected to SDS/PAGE (Fig. 2 *Inset*). One set was stained with Coomassie blue and a companion set was used for Western blotting with antiserum C. The apparent molecular weight of the α' carboxyl-terminal-reacting material, as inferred from the known molecular weights of lamprey albumin and the component fibrinogen chains used as markers, was 35,000–40,000. It was eluted from the G-100 column behind the easily visualized fragment E, the molecular weight of which is known to be 45,000. The migration of the α' component was only slightly retarded by reduction, indicating intrachain disulfide bonding but no interchain attachments. The material did not react with antiserum B, which was made against residues 208–218.

Affinity Chromatography. Typically, 15 mg of lamprey fibrinogen that had been lightly digested with trypsin was passed over a 2-ml Gly-Pro-Arg affinity column at 4°C. After washing with an equal volume of the starting buffer, elution of the bound fragment D was accomplished with 1.0 M NaBr/0.05 M sodium acetate (pH 5.3). Fractions were read at 280 nm and appropriately pooled (Fig. 3). Pools were freeze-dried and dissolved in 8 M urea/2% SDS before being run on SDS/polyacrylamide gels. Gel patterns were consistent with fragment E and other material passing directly through the column, and fragment D being completely bound (Fig. 3 *Inset*).

Western blotting with antiserum C showed that the material corresponding to the α' chain did not bind to the Gly-Pro-Arg column, all of it appearing in the dropthrough as a component of apparent molecular weight 35,000–40,000 (Fig. 3 *Inset*). As in the case of the gel filtration experiments, the minimal effect of reduction on the position of the band implied the existence of intrapeptide but no interpeptide disulfide bonds. The size of the fragment suggests that the major trypsin attack point is situated somewhere between Lys-308 and Arg-341. In other experiments, the α' fragment did not bind to other potential affinity columns, including a Gly-Val-Arg support that mimics the lamprey fibrin β chain (data not shown).

Incorporation of α' Chains into Fibrin. Lamprey fibrinogen was clotted with either lamprey or bovine thrombin under conditions appropriate (or not) for the activation of latent factor XIII. Aliquots were subjected to SDS/PAGE on paired gels, one of which was stained with Coomassie blue, and the other used for Western blotting with either antiserum C or antiserum B. In all cases the monomeric form of the α' chain disappeared rapidly, none being detectable after 10 min when clots formed in about a minute. Surprisingly, only antiserum B

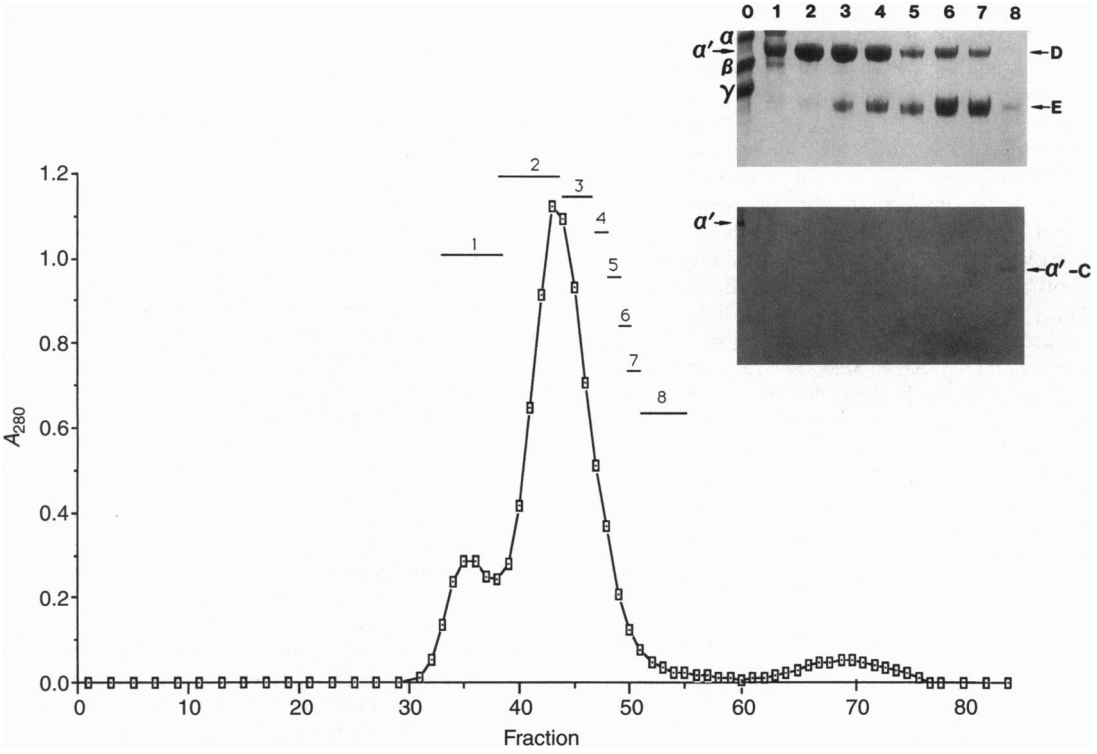

FIG. 2. Gel filtration of light tryptic digests of lamprey fibrinogen on a Sephadex G-100 column (2.5 cm × 70 cm, equilibrated and developed with 0.3 M NaCl; 3.0-ml fractions). (*Inset*) SDS/polyacrylamide minigel electophoresis of fractions. Coomassie blue staining (*Upper*) and Western blot with antiserum C (*Lower*) are shown. Note that the α′-containing fractions were eluted behind fragment E (molecular weight, 45,000). Lane 0, reduced lamprey fibrinogen; lanes 1–8, pools from gel filtration column (samples were not reduced).

clearly revealed the rapid appearance of dimers and, in the later stages, some higher polymers. In contrast, antiserum C was only minimally effective (Fig. 4). At first we thought that incipient proteolysis was removing the carboxyl-terminal domain with which antiserum C reacts, but experiments in which phenylmethanesulfonyl fluoride was added 10 min after thrombin addition eliminated all evidence of proteolysis but still resulted in minimal detection of the dimer by antiserum C. Rather, it appears that the carboxyl-terminal segment itself is involved in covalent crosslinking and is restricted from interacting with the antibody as a result.

The results with antiserum B show that the α′ chains were crosslinked into dimers about as rapidly as were γ chains (Fig. 4). The α′-chain crosslinking cannot be intramolecular, because it is inhibited by peptides that prevent polymerization (Fig. 5, lanes 7–12), the inhibition occurring to the same extent as the inhibition of γ dimer formation (Fig. 5, lanes 7–12). In other experiments we showed that both kinds of crosslinking were also inhibited to the same extent by glycine ethyl ester and other amines known to prevent transamidation by factor XIII.

Fortuitously, in some of these experiments antiserum C was at such a dilution that it crossreacted with ordinary α chains,

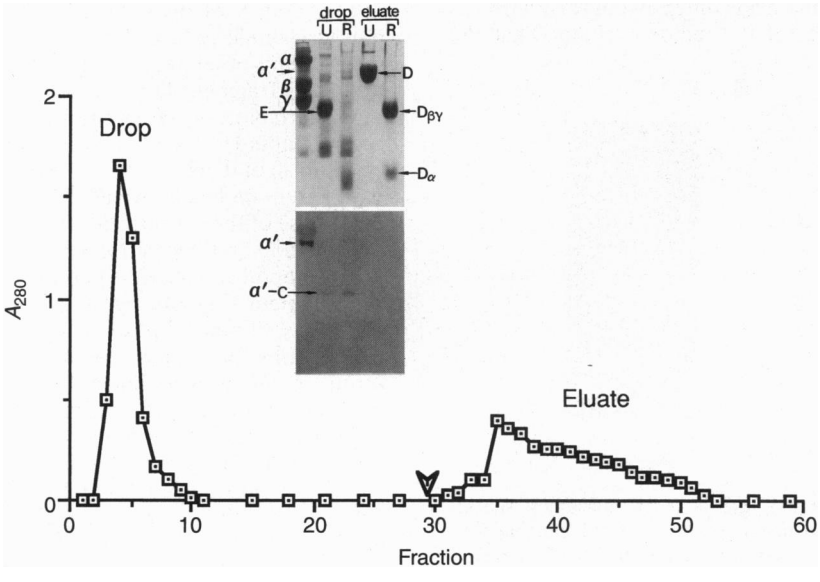

FIG. 3. Affinity chromatography of light tryptic digests of lamprey fibrinogen on a Gly-Pro-Arg column, showing that the α′ carboxyl terminus does not bind under the same conditions as does fragment D. Arrowhead denotes beginning of NaBr elution. (*Inset*) SDS/polyacrylamide minigel electrophoresis of fractions. Coomassie blue staining (*Upper*) and Western blot with antiserum C (*Lower*). U, unreduced; R, reduced.

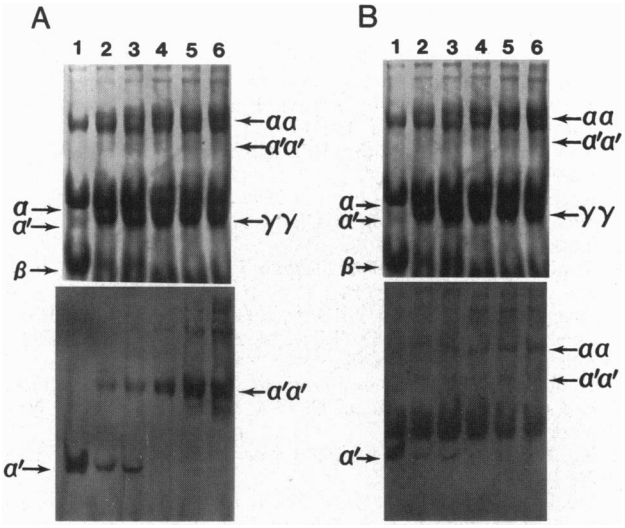

FIG. 4. Demonstration of rapid crosslinking of α' chains during clotting. Lamprey fibrin preparations were clotted with bovine thrombin in the presence of Ca^{2+} and cysteine, and reduced samples were analyzed by SDS/polyacrylamide minigel electrophoresis (3.3 hr, 50 V). Samples were poisoned at intervals by the addition of an equal volume of 8 M urea/2% SDS. Time points read from left to right: lanes 1–6 correspond to 0, 5, 10, 30, 60, and 150 min, respectively. (*Upper*) Gels stained with Coomassie blue. (*Lower*) Western blots with antiserum B (*A*) or antiserum C (*B*).

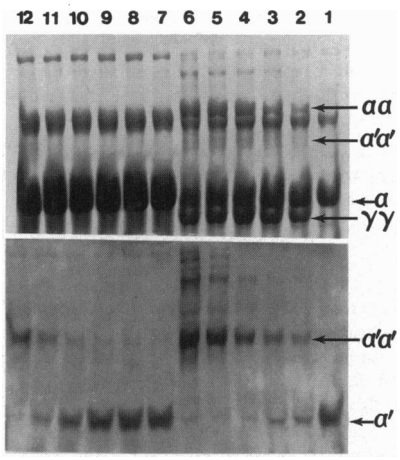

FIG. 5. Polymerization is required for crosslinking of α' chains. Lamprey fibrin preparations were clotted with bovine thrombin in the presence of calcium ions and cysteine, and reduced samples were analyzed by SDS/polyacrylamide minigel electrophoresis (3.3 hr, 50 V). Samples were poisoned at intervals by the addition of an equal volume of 8 M urea/2% SDS. Time points read from right to left: lanes 1–6 and 7–12 correspond to 0, 5, 10, 30, 60, and 150 min, respectively. Samples in lanes 7–12 contained 5 mM Gly-Pro-Arg-Pro-amide as an inhibitor of polymerization. (*Upper*) Coomassie blue staining. (*Lower*) Western blotting with antiserum B.

so that heterodimers could be distinguished from homodimers (Fig. 4). In this regard, there was no indication of any α–α' heterodimers, all the crosslinks being between α' chains, and to a much lesser extent, homodimerically between ordinary α chains.

DISCUSSION

Lampreys are the earliest diverging animals known to have a thrombin-activated fibrinogen. Most of the features of the molecule have parallels in human fibrinogen, including subunit constitution, fibrinopeptide release, and the inhibition of polymerization by Gly-Pro-Arg derivatives (13). The sequences of both the β and γ chains of lamprey and human fibrinogens are about 50% identical; the major forms of α chain are similar over the course of the first 150 amino acids or so, but are then very different for the remainder, including different repetitive sequences (12).

Structural Aspects. The discovery of an α chain in lampreys with a carboxyl-terminal segment homologous to those of β and γ chains was not altogether unexpected (1), since it had already been hypothesized that the archetype of vertebrate fibrinogen must have been composed of identical multidomainal chains whose amino and carboxyl portions were derived from separate sources (14). As it happens, domains sharing common ancestry with the carboxyl-terminal domains of β and γ chains are found in numerous other settings (reviewed in ref. 15). When the sequences of these domains are aligned and subjected to phylogenetic analysis, the minor form, α' domains of lamprey, chicken, and human fibrinogens invariably cluster together. This is true even though in lampreys the α' chain is encoded by a separate gene (1), but in the human (2) and chicken (3) it is the result of an alternative splicing arrangement.

Recent observations on the equivalent human fibrinogen (5) indicate that the molecules appear never to be heteromeric with regard to the two kinds of α chain. The same situation seems to occur in lampreys, as indicated by the single component observed by Western blotting in the presence of two components of ordinary fibrinogen. In the latter case, the faster moving component is thought to be due to degraded α chains, which upon reduction move to the same position as β chains (Fig. 1). The occurrence of symmetric dimers in lamprey fibrinogen is perhaps less surprising since the joining point for the dimerization of half-molecules is different for the two kinds of α chain, whereas in humans, because the alternative chains are the result of splicing differences, the joining points are exactly the same.

It is also clear that in lampreys the α' chains are not disulfide-linked through their carboxyl-terminal domains, in contrast to what has been suggested by others for the human minor α chain (5). Given that the cysteine distribution in the lamprey protein is exactly the same as occurs in the chicken and human α' homologues, it seems unlikely that any of these is disulfide-linked within their parent molecules. There is no evidence, either, that the lamprey domains are intramolecularly associated; certainly they are unassociated after proteolytic removal from the parent molecule. Electron microscopy of purified preparations of α' fibrinogen may settle the point.

Functional Aspects. The alternative α chain has been conserved over the course of vertebrate evolution, from lampreys to mammals, the last common ancestor of which occurred about 450 million years ago. As such, it must have an important function. One role of the α' chain and its primitive carboxyl-terminal domain may be involvement in the association of protofibrils. Protofibrils are intermediate polymers composed of 15–20 fibrin monomeric units held together by knob–hole interactions involving Gly-Pro-Arg (16). Gelation ensues upon the extensive association of those protofibrils. The involvement of α' chains in protofibril formation would be consistent with their relatively low abundance, which amounts to only one to two molecules per protofibril. Under this proposal, the flexibly attached carboxyl domains of the α' chains would interact with similar domains in other protofibrils, thereby accelerating this stage of polymerization. This would account for the rapid crosslinking of the α' chains when clotting occurs under native conditions where factor XIII is active. The α' dimers appear almost as quickly as γ-chain dimers and much more rapidly than dimers of ordinary α chains. Other workers have proposed that in mammals the association of protofibrils involves the association of (ordinary) α chains (17). Such a proposal seems inconsistent with the very slow and imprecise

formation of factor XIII-introduced crosslinks into these abundant α chains, however.

In summary, the lamprey alternative α chain, now referred to as α', occurs in plasma fibrinogen at a level of 5–10% of the ordinary type. Molecules containing this chain are rapidly crosslinked during clotting, at a rate comparable to γ chains and much faster than ordinary α chains. Although they have a carboxyl-terminal domain similar to β and γ chains, these domains do not bind to Gly-Pro-Arg or Gly-Val-Arg peptide columns.

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