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Biochemistry

Fib_{420}: A normal human variant of fibrinogen with two extended α chains

(clotting factor/blood plasma/fibrinogen α chain)

YIPING FU AND GERD GRIENINGER*

The L.F. Kimball Research Institute of The New York Blood Center, 310 East 67th Street, New York, NY 10021

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ABSTRACT **In fibrinogen, α_E chains form a subpopulation of α subunits that are distinguished by a carboxyl extension homologous to the C termini of the other two constituent chains: β and γ. The molecular mass of α_E is >50% greater than that of the common α subunit, due in part to an extra 236 amino acids. These residues are encoded by exon VI, a recently discovered extension of the fibrinogen α gene. Additional mass is contributed by posttranslational processing, including N-glycosylation, which, based on experiments with the inhibitor tunicamycin, was found to account in large measure for α_E migration on SDS/PAGE at ≈110 kDa rather than at its calculated mass of 92,843 Da. An antibody specific for the exon VI-encoded domain of α_E (anti-VI) and capable of recognizing α_E-containing fibrinogen in both native and denatured form was generated using a recombinant protein as immunogen. Its use in Western blot analysis of fractions of normal human blood (plasma and preparations of fibrinogen) revealed a single, sharp, α_E-containing band migrating behind the position of the broad, predominant fibrinogen band, $(\alpha\beta\gamma)_2$. Designation of the upper band as Fib_{420}, an ≈420-kDa homodimer of the formula $(\alpha_E\beta\gamma)_2$, is based on the overwhelming proportion of α_E subunits (>80% of the total α chains) found in anti-VI-immunoprecipitable material from hepatoma cell medium. Several lines of evidence suggest that the α_E subunit, alone or incorporated into fibrinogen, is more stable than the common α chain, a feature of potential clinical importance.**

The major clotting protein, fibrinogen, is composed of paired sets of three subunits (α, β, and γ) that are encoded on separate genes (1, 2). The β and γ subunits display strong homology between their C-terminal domains, which is not shared by the α subunit in its most abundant form. However, recent discovery of an extra exon (exon VI) in the fibrinogen α gene has led to identification of an extended α subunit (α_E), a naturally occurring species with a strikingly β- and γ-like globular C terminus (3). The first evidence for the exon, which encodes the 236 amino acids of the carboxyl extension, was an α-chain transcript in normal liver that is "bipartite"—i.e., its α coding region is followed by an untranslated region and a long, second open reading frame (4). Additional splicing of the bipartite transcript—removal of the untranslated region (intron E, separating exons V and VI)—leads to expression of the extra sequence as the C-terminal domain of α_E. In contrast, the predominant α chain, which lacks this homology to the β and γ chains, derives its C terminus from translation of 14 codons into intron E.

Using human hepatoma (HepG2) cells as well as transfected COS cells, it has been shown that α_E is assembled into fibrinogen molecules (3). Based on studies using a specific antibody to the exon VI-encoded carboxyl extension, we now report the presence of the α_E chain in a fibrinogen variant of human blood. We show that most of the variant molecules have α_E in place of both α chains to produce a significantly larger than normal fibrinogen species.†

MATERIALS AND METHODS

A large segment of the exon VI-encoded region of α_E, from Gly-635 to the terminal Gln-847, was expressed in *Escherichia coli* using vector pQE-9 (Qia*express*; Qiagen) as described (3). A highly specific antibody against the urea-denatured recombinant protein was produced in rabbits and is referred to as anti-VI.

All human hepatoma cell lines—HepG2 and Hep3B2 (5), PLC/PRF/5 (6), and HuH7 (7)—as well as COS-1 cells were cultured and labeled as described (3, 8). Vectors (pBC12BI) containing full-length cDNAs for α_E and α and the transfection procedures have been described (3, 8).

Human fibrinogen was purchased from American Diagnostica (Greenwich, CT) and from Sigma or prepared as described (9) from the pooled plasma of normal blood donors. For Western blot analysis, fibrinogen or its subunits were separated by SDS/7.5% PAGE and transferred onto 0.2-μm nitrocellulose membranes (Bio-Rad). The primary antibodies, produced in rabbits, were either anti-fibrinogen (antibody to whole human fibrinogen; Dako) or anti-VI. The secondary antibody was a goat anti-rabbit IgG (colloidal gold system; Bio-Rad).

RESULTS

Mature α_E contains 847 amino acids (3). Its predicted molecular mass of 92,843 Da corresponds well with the size estimates from SDS/PAGE analysis of intracellular fibrinogen in HepG2 cells and transfected COS cells. In secreted fibrinogen, however, α_E migrates with an increased molecular mass of ≈110 kDa, indicating a significant degree of processing. To evaluate N-glycosylation of α_E (its elongated C terminus contributes two potential sites), we performed experiments with the inhibitor tunicamycin (Fig. 1). Relative to the controls, α_E synthesized in the presence of tunicamycin migrated significantly faster (with an apparent molecular mass of about 95 kDa), suggesting that N-glycosylation accounts for most of the increased mass evident upon secretion. As expected, the same pattern was observed for the β and γ chains but not for the predominant α chain, due to its well-documented lack of carbohydrate (11).

By Western blot analysis with an antibody against the exon VI-encoded domain of α_E (anti-VI), it was possible to demonstrate the presence of α_E chains in normal human blood

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Abbreviation: αC, C terminus of the common α chain; α_E, extended α subunit of fibrinogen.

*To whom reprint requests should be addressed.

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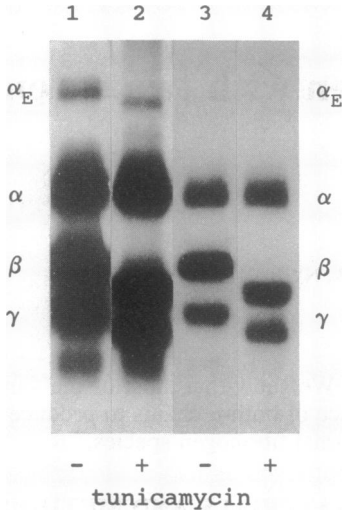

FIG. 1. N-glycosylation of α_E: susceptibility of α_E synthesis to tunicamycin. HepG2 cells were cultured for 24 h in the presence of recombinant, baculovirus-derived human interleukin 6 at 20 ng/ml (10). Thereafter the cells were incubated for 2 h with [^{35}S]methionine in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of tunicamycin (Sigma). Treatment of monolayers with tunicamycin was initiated 15 h before radioactive labeling by adding the inhibitor (5 μg/ml) to the medium and maintaining it in all subsequent wash and labeling procedures. Fibrinogen secreted into the culture medium was immunoprecipitated with antibody to whole human fibrinogen. The immunoprecipitates were separated by SDS/10% PAGE under reducing conditions and detected by autoradiography. Lanes 1 and 2 were overexposed to make the α_E subunits visible. Positions of the individual fibrinogen chains are indicated.

(Fig. 2). Analysis of both human plasma and purified human fibrinogen, separated by SDS/PAGE under reducing conditions, revealed a single band at ≈110 kDa (Fig. 2*A*). Identical results were obtained with preparations of fibrinogen from different sources (data not shown). Virtually no crossreaction of anti-VI with the β and γ chains of fibrinogen was detectable. Under nonreducing conditions (Fig. 2*B*), α_E-containing fibrinogen was confined to a single, sharp band migrating behind the broad predominant fibrinogen band. The latter is composed of 340-kDa molecules and their 305- and 270-kDa degradation products (12, 13).

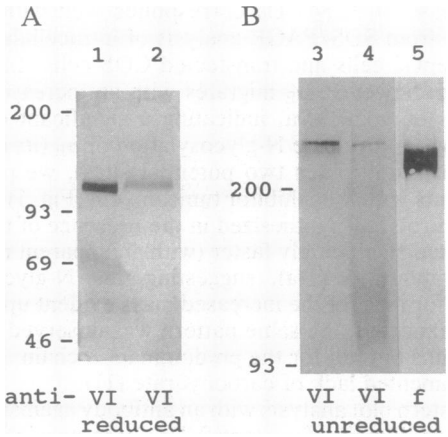

FIG. 2. Presence of α_E-containing fibrinogen in human blood: Western blot analysis using anti-VI. Fibrinogen was separated by SDS/7.5% PAGE under reducing (*A*) and nonreducing (*B*) conditions and detected by Western blot analysis with either anti-fibrinogen (f) or anti-VI as indicated. Lanes 1 and 3 contained 10 μg and lane 5 contained 0.1 μg of purified fibrinogen (American Diagnostica, Greenwich, CT); in lanes 2 and 4, normal human plasma (2.5 μl) was applied. Sizes of protein markers are indicated in kDa.

To further analyze this large, α_E-containing fibrinogen molecule, we explored its subunit stoichiometry. Immunoprecipitates of material secreted by HepG2 cells were examined on SDS/PAGE gels under disulfide-reducing conditions so that comparison of subunits could be made between those obtained with anti-fibrinogen and those obtained with anti-VI (Fig. 3*A*). From lanes 1 and 2, generated by immunoprecipitation with anti-fibrinogen, α_E was estimated to represent ≈5% of the total number of α chains. This percentage, which reflects the relative rate of α_E synthesis in these hepatoma cells, is comparable to the level of α_E transcripts in normal human liver estimated by reverse transcription-PCR and Northern blot analysis (3). Given such a low ratio, it came as a total surprise that the α chain population of the fibrinogen immunoprecipitated with anti-VI (lane 3) was >80% α_E. Similar results were found in other human hepatoma cell lines: PLC/PRF/5, HuH7, and Hep3B2 (data not shown). Analysis of the HepG2 immunoprecipitates under nonreducing conditions confirmed localization of the secreted α_E subunits to high molecular mass, disulfide-linked complexes (Fig. 3*B*). No free α_E chains were detected, indicating that HepG2 cells, like heterologous COS cells (3), do not secrete significant amounts of unassembled α_E subunits.

These results suggest that α_E-containing fibrinogen is a symmetrical molecule of the general formula $(\alpha_E\beta\gamma)_2$. Based on the estimate of ≈110 kDa for the individual α_E chain (see Fig. 2 and ref. 3), we calculate the molecular mass of this fibrinogen variant to be ≈420 kDa and have therefore termed it Fib_{420} to distinguish it from the abundant 340-kDa form of fibrinogen, denoted Fib_{340}: $(\alpha\beta\gamma)_2$. It is not clear whether the low level of common α chains detected in the anti-VI immunoprecipitates (Fig. 3*A*, lane 3) is due to coprecipitation of

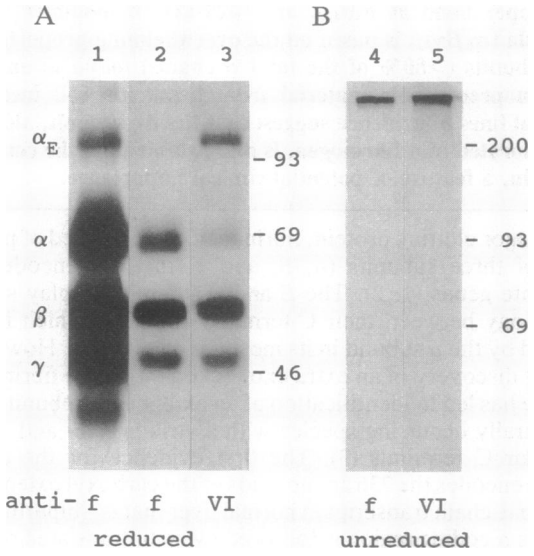

FIG. 3. Stoichiometry of α_E-containing fibrinogen: immunoprecipitation of α_E-containing fibrinogen with anti-VI. HepG2 cells were cultured with interleukin 6 as described in Fig. 1. After incubating the cells for 2 h with [^{35}S]methionine, the culture medium was immunoprecipitated with either anti-fibrinogen (f; lanes 1, 2, and 4) or anti-VI (lanes 3 and 5). The immunoprecipitates were separated by SDS/10% PAGE under reducing (*A*) or nonreducing (*B*) conditions and detected by autoradiography. For the purpose of quantitation, lanes from multiple autoradiograph exposures were scanned by densitometry, and values obtained for the individual fibrinogen α subunits were corrected for methionine content. Positions of the individual fibrinogen chains are indicated. In the composite shown, lane 2 was underexposed for better comparison with lane 3. Exposures of lanes 4 and 5 were similarly adjusted. Proteolysis that gives rise to the broad predominant fibrinogen band in plasma (see Fig. 2*B*) does not occur in the cell culture medium under the described conditions.

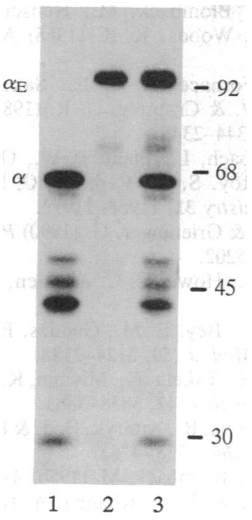

FIG. 4. Differential stability of α_E and α: transfected COS cells as a model. COS-1 cells were transiently transfected with pBC12BI vectors containing α and/or α_E cDNAs. As with the cells in Fig. 3A, they were labeled, and cell extracts were immunoprecipitated with anti-fibrinogen prior to analysis by SDS/PAGE. Lane 1, α; lane 2, α_E; lane 3, α plus α_E. Protein markers are indicated on the right in kDa.

Fib_{340} or to the existence of a population of asymmetrical molecules that bear one α_E and one α chain.

Heterogeneity of the predominant plasma fibrinogen form, evidenced by the broad band it yields on SDS/PAGE gels (see Fig. 2, lane 5), reflects degradation of the C-terminal domain of its component α subunits (αC) in the circulation (12). By comparison, the lack of heterogeneity in the bands of both α_E and Fib_{420} on SDS/PAGE gels (Fig. 2 *A* and *B*, respectively) suggests that they are less susceptible to proteolysis. To evaluate whether the presence of the exon VI-derived domain protects the αC domain of individual α_E chains, we examined immunoprecipitates of extracts from COS cells transfected with α and/or α_E cDNAs (Fig. 4). Under conditions in which nearly 50% of the common α chains (67 kDa) were degraded to lower molecular mass peptides, the amount of intact α_E chains remained virtually undiminished, which provides clear evidence that α_E chains on their own are more stable than the predominant α-chain species.

DISCUSSION

This study shows that the elongated α-chain species, known as α_E, is a component of circulating human fibrinogen. The α_E-containing fibrinogen molecules are primarily of the structure $(\alpha_E\beta\gamma)_2$ and have a calculated molecular mass of $\approx$420 kDa, based on an estimate of $\approx$110 kDa for each α_E chain. The relatively low circulating level of this larger fibrinogen molecule, referred to as Fib_{420}, is undoubtedly responsible for its having escaped detection until now despite numerous studies on the structure and function of fibrinogen. It remains to be seen whether any of the functional characteristics previously attributed to Fib_{340} are in fact due to the presence of low levels of the Fib_{420} variant in the fibrinogen preparations commonly studied.

Preliminary estimates by ELISA place the concentration of α_E in normal plasma at approximately 1–2% that of the predominant α chain (unpublished results), which is comparable to their relative mRNA levels in normal human liver (3) and close to the relative rate of α_E synthesis (5%) in HepG2 cells (Fig. 3). If assembly of α_E chains into fibrinogen were a stochastic process, as appears to be the case with the γ and γ' chain variants (14), the occurrence of symmetrical molecules with two α_E subunits should be very low (2.5% or less of all α_E-containing fibrinogen molecules). The fact that the α-subunit content of anti-VI-immunoprecipitable fibrinogen is >80% α_E suggests instead a highly selective assembly process, involving perhaps a specific chaperone or a thermodynamically favored symmetrical conformation. The latter mechanism appears to hold for fibrinogen molecules from a number of heterozygous individuals with congenital fibrinogen α chain defects. In the circulation of these patients, symmetrical molecules, both normal and abnormal, but no hybrids are found (15). The driving force for the selectivity in several cases may be formation of an additional disulfide bond between the mutant chains (16, 17). Although only intrachain bonds of this type have been found among the homologous cysteine residues in the β- and γ-chain C termini, interchain bonds involving the four exon VI-encoded cysteines of α_E cannot *a priori* be excluded.

Like its counterparts in most other species, the common α chain of human fibrinogen contains no carbohydrate moiety. In contrast, the α_E chain is N-glycosylated (Fig. 1). Its elongated C terminus contributes two potential sites, at Asn-667 and Asn-812. Of these, the tripeptide consensus sequence at Asn-667 is conserved in all vertebrate α_E counterparts down to a homologous second α chain in lamprey (4, 18) and is most likely used. It remains to be seen whether glycosylation also occurs at Asn-812, a site that is conserved only in mammals (unpublished results).

Several lines of evidence suggest that the α_E chain is less susceptible to proteolytic degradation than the common α chain. First, Fib_{420} is homogeneous; it forms a sharp band on SDS/PAGE (Fig. 2*B*). Second, α_E derived from plasma fibrinogen by reduction migrates as a single band (Fig. 2*A*). Third, when the stability of unassembled, single α chains is studied in the COS cell system, considerable degradation of common α is observed while most of α_E remains intact (Fig. 4). It is particularly noteworthy that little or no cleavage occurs at the Val-610/Arg-611 site in α_E, as evidenced by the fact that with anti-VI we do not detect a 43-kDa cleavage product (Fig. 2). It is well known that in the common α chain, presumably as a result of cleavage at the corresponding site (19), the C-terminal 15 amino acids (positions 611–625) are somehow removed prior to the isolation of fibrinogen from plasma. Resistance of α_E to such cleavage could be conferred by either sequence differences downstream from the site or by hindered access for proteases created by the large, globular, exon VI-encoded domain.

A tentative model of Fib_{420} is schematically presented in Fig. 5. It shares several key features with other models of fibrinogen structure: a central nodule (or disulfide knot containing the N termini of all six chains) from which emanate two arms (the coiled coils, composed of the α-helical regions of each set of α, β, and γ chains) and two distal nodules formed by the highly homologous β and γ C-terminal domains. Although some models of fibrinogen structure depict the αC domains as free-swimming appendages (20, 23), others show them folding back to the central region (24–26). We patterned our model for Fib_{420} after the latter, based on several electron microscopy studies. In these studies, a fourth nodule associated with the central sphere was detected at low frequency (21, 27, 28). This fourth nodule appears to be related to the C-terminal end of the α chain (27) and is seen on <10% of the molecules (22), which is suggestively close to the level of Fib_{420} in most fibrinogen preparations. As the model suggests, these tetranodular forms may indeed be molecules of Fib_{420}, with the fourth nodule created by the two identical exon VI-encoded domains of the α_E chains. In addition to forming a structure that should increase the inaccessibility of α_E chains to proteases, the model allows for possible disulfide bond formation between the α_E C termini,

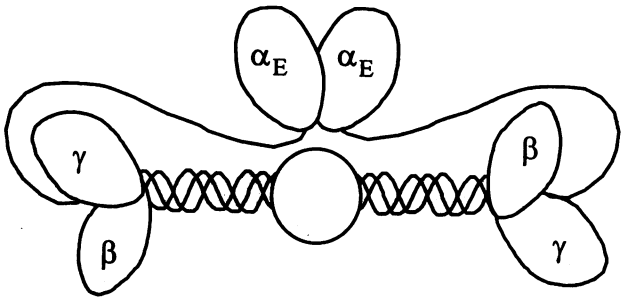

FIG. 5. Schematic representation of Fib_{420}. The molecule is shown as a tetranodular structure, modified after models of fibrinogen proposed by different authors (20–22). The Fib_{420} hexamer contains two sets of three chains (α_E, β, and γ) interlinked by disulfide bonds. The amino ends of all six chains are contained in the central sphere. Diverging from this domain are two triple-chain coiled coils that connect to the two distal nodules, each containing as subdomains the C termini of the β and γ chains. Extending from these are the long αC domains of the α_E chains that fold back to the central region, bringing the exon VI-encoded C termini together to form a fourth nodule. Each chain is labeled in its globular domain.

discussed above, which serves as a plausible driving force in the assembly of symmetrical molecules.

The Fib_{420} variant's two extra globular domains, whatever their actual position may be, are likely to bear significantly on the fibrinogen molecule's multiple polymerization sites and binding/adhesion capacities. At present, little is known about the physiological role of Fib_{420}. Enhancement of α_E synthesis by interleukin 6 suggests that Fib_{420} participates in both normal physiology and the acute-phase response (3). Recent Western blots of thrombin-treated fibrinogen using anti-VI provide evidence of the participation of Fib_{420} in fibrin gel formation (unpublished results). The fact that α_E deficiency is not lethal can be derived from a recent report on fibrinogen Marburg, a homozygous case of dysfibrinogenemia (29). In the α gene coding for this abnormal fibrinogen, a single-base substitution (A → T) has been identified that changes AAA (lysine) to TAA (stop) at codon 461. As a result, the C-terminal segment 461–625 of the common α chain is lacking, and formation of α_E is not possible. Symptoms displayed by the homozygous propositus consisted of severe hemorrhage after delivery followed by repeated thrombotic events that occur, paradoxically, despite unusually low fibrinogen levels. Further analysis of the effects of altered α_E production should clarify the specific contribution of Fib_{420} to fibrinogen metabolism.

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