Assembly and Secretion of Fibrinogen

INVolvEMENT OF AMINO-TERmINAL DOMAINS IN DIMER FORMATION*

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Fibrinogen is a dimer with each half-molecule composed of three different chains (Aα, Bβ, γ). Previous studies showed that amino-terminal disulfide bonds, as well as the disulfide rings that flank the “coiled-coil” region, are necessary for chain assembly and secretion (Zhang, J. Z., and Redman, C. M. (1994) J. Biol. Chem. 269, 652–658). We now determine whether other amino-terminal domains are involved in linking the half-molecules. Fibrinogen chains, with deletions at the amino terminus, were co-expressed in COS cells together with normal fibrinogen chains. Elimination of the first 8 amino acids of the Bβ chain did not affect dimer assembly, but deletion of amino acid residues 9–72 had a small inhibitory effect on dimer formation. Deletion of the first 72 amino acids of the Bβ chain further inhibited dimer formation and resulted in nearly equal amounts of half-molecule and dimeric fibrinogen being formed and secreted. Deletion of the first 80 residues, which includes the cysteine residues that form the amino-terminal disulfide ring, completely eliminated dimer formation, and only half-molecules were secreted. By contrast, deletion of the first 41 amino acid residues of the Aα chain or the first 15 residues of the γ chain, which correspond to Bβα1–72, did not affect chain assembly and secretion. However, co-expression of both Aαα1–41 and γγα1–15 with normal Bβ, inhibited dimer formation. Taken together, these results indicate that in addition to disulfide bonds, noncovalent interactions of other amino-terminal amino acid residues in the three fibrinogen chains also participate in dimer formation.

Human fibrinogen is a dimer with each half-molecule containing three different polypeptide chains Aα, Bβ, and γ (for reviews, see Refs. 1–3). Each of the fibrinogen chains has an amino-terminal segment followed by an α-helical domain of about 111 amino acids that forms a 3-chain “coiled-coil” region flanked by interchain disulfide rings (4). On the carboxyl-terminal end, the Bβ and γ chains have homologous globular domains, while the Aα chain has a long segment that is not equivalent to that of Bβ and γ. However, in normal plasma there are small amounts of a fibrinogen with an extended Aα chain, which is homologous to the carboxyl termini of the Bβ and γ chains (5). Structural studies indicate that fibrinogen is elongated and trinodal. The central node (E domain) contains the amino termini of the 6 polypeptide chains, and the two terminal nodes (D domains) are formed by globular carboxyl-terminal domains of Bβ and γ chains. The carboxyl-terminal domain of the large Aα chain is thought to fold back and contribute to the structure of the central node (12–16). The fibrinogen chains are held together by 29 inter- and intra-chain disulfide bonds (6–11).

Previous studies showed that intracellular assembly of the 2 half-molecules into a dimer requires not only the amino-terminal disulfide bonds but also that the disulfide rings that flank the coiled-coil region remain intact (17–19). Disruption of the disulfide rings at the amino-terminal end of the coiled-coil region prevents dimer formation, and the elimination of the disulfide rings, at the carboxyl-terminal end of the coiled-coil region, allows dimer formation, but the 6-chain molecule that is assembled is not secreted (19). To determine whether other amino-terminal domains are involved in fibrinogen assembly, we have constructed a series of deletion mutants and transiently co-expressed the mutant chains in COS cells together with two other normal or mutant chains. The half-molecules and dimeric fibrinogen, formed intracellularly and secreted into the medium, were determined.

EXPERIMENTAL PROCEDURES

Materials—Full-length Aα, Bβ, and γ cDNAs, cloned into the PstI site of pBR322, were kind gifts from Dr. Dominic Chung (University of Washington, Seattle). Expression vector pE4-Neo (20) was obtained from the Genetics Institute (Cambridge, MA). Other reagents used have been described (17, 21–24).

Plasmid Construction and Mutagenesis—Full-length Aα, Bβ, and γ cDNAs were subcloned into M13mp18 or M13mp19 (17–19). To construct pE4-NeoAα, pE4-NeoBβ, and pE4-Neoγ, EcoRI sites were introduced into the 5’ end of Aα and Bβ cDNA of M13mp19Aα, the 5’ side of PstI of M13mp18Bβ, and the 5’ and 3’ ends of γ cDNA of M13mp18γ by site-directed mutagenesis (25, 26, 28). The following oligonucleotides were used: CTTTCTAACATTTGGCTGCTC, GACCTGCAAGAACTCAGCTATC to change the nucleotide sequences GAA to GAG without changing glutamic acid at position 550. Each of the full-length cDNAs was excised by digestion with EcoRI and inserted into the EcoRI site of expression vector pE4-Neo. The correct orientation was selected by restriction mapping.

Mutants of Aα—Cysteine codons (TGC) in single strand DNA of M13mp19Aα were changed to serine (AGC) at positions 28 and 36. Two synthetic oligonucleotides, TGAATTTCTCGTGGCAAGAGATTAA at position 28 and TCATCGAGAAGAGAGGAGCCA for Aα28s and TCATCGAGAAGAGAGGAGCCA for Aα36s were used according to the method of Kunkel (25). The mutations were confirmed by DNA sequencing (27). A deletion mutant Aαα1–41, was constructed using the oligonucleotide GAAAGGGGTTATGGAGTAGATCCTGAGGTAACGTGAGG (17). The mutant was screened by digestion with EcoRI and confirmed by DNA sequencing. All mutant cDNAs were released by digestion with EcoRI and ligated into the EcoRI site of pE4-Neo. The proper orientation of mutant cDNAs and the mutations formed by simple base substitutions were confirmed by DNA sequencing.

Mutants of Bβ—Four deletion mutants M13mp19Bβα1–8, M13mp19Bβα19–72, M13mp19Bβα1–80, and M13mp19Bβα1–192 were prepared as described previously (17). The oligonucleotides used for
Expression of Individual Mutant Chains—All normal and mutant fibrinogen chain cDNAs were subcloned into the expression vector pED4Neo. The vector was chosen because we anticipate, in future studies, selecting stable cell lines and also because it gives high levels of expression in COS cells. This vector contains TfR neo neomycin phosphotransferase (Neo), a selectable marker, and dehydrofolate reductase, an amplifiable gene. pED4Neo also has the SV40 origin of replication and enhancer and the adenovirus major late promoter (20). Forty hours after transfection, COS cells were radiolabeled with L-[35S]methionine for 2 h, and the radioactive fibrinogen chains were immunoprecipitated from both the detergent-treated cell lysate and from the culture medium, using a polyclonal antibody that recognizes the three fibrinogen chains. The radioactive chains were separated by SDS-PAGE and detected by autoradiography.

RESULTS

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A diagram, depicting the deletion mutants constructed, is shown in Fig. 1. All normal and mutant chains were expressed to approximately the same extent (Fig. 2). Deletion mutant Bβ1–192 was less radioactive, which could be due to fewer methionine residues (Fig. 2A, lane 9). Lower molecular weight proteins, due to either degradation or to incomplete translation, were noted in normal and mutant Aα and Bβ chains (Fig. 2A).

To determine whether single mutant chains are secreted, the culture medium was analyzed. A very small amount of normal Aα, Aα2Bβ8,36, and Aα1–41 were secreted (Fig. 2B, lanes 1, 2, and 3). Larger amounts of normal γ and γ1–15 were secreted (Fig. 2B, lanes 10 and 11). There was no secretion of normal Bβ, Bβ1–8, Bβ9–72, Bβ1–72, Bβ1–80, and Bβ1–192 chains. In all cases the secreted chains were only detected on overlap of the autoradiogram, and we estimate that less than 10% of the γ chain synthesized was secreted into the medium. The large amount of protein radioactivity at the top of the gel is a contaminant due to cross-reaction of the polyclonal antibody with a protein secreted by COS cells.

Role of Amino-terminal Domain of Bβ Chain in Dimer Formation—Our previous study showed that deletion of the first 72 amino acids from Bβ chain inhibits assembly of the two half-molecules and results in secretion of both half-molecules and dimeric fibrinogen (17). To more narrowly define the domains involved in assembly of dimeric fibrinogen, Bβ mutants with different deletions at the amino terminus were constructed and co-expressed with normal Aα and γ chains. The intracellular complexes were analyzed by immuno-precipitation and SDS-PAGE on nonreduced gels. Secretion was measured by immunoprecipitation of fibrinogen from the culture medium and analysis of the secreted fibrinogen chain complexes on nonreduced 5% SDS-PAGE or on reduced 7.5% SDS-PAGE.

COS cells co-expressing Bβ1–8 with normal Aα and γ chains secreted the mutant dimeric fibrinogen and a small amount of Aαγ complex (Fig. 3A, lane 1). On reduction the component chains of fibrinogen were observed (Fig. 3B, lane 1). Free chains, intermediate complexes, and dimeric fibrinogen occurred intracellularly (data not shown). Expression of a mutant Bβ chain with an internal deletion of amino-terminal residues 9–72, which is the same as the naturally occurring mutant termed Fibrinogen New York 1 (29), allowed assembly and secretion of dimeric fibrinogen and small amounts of half-molecules and of Aαγ complex (Fig. 3A, lane 2). On reduction Bβ9–72 co-migrates, on SDS-PAGE, together with the γ chain (Fig. 3B, lane 2). However, deletion of the first 72 amino acid residues (Bβ1–72) resulted in inhibition of dimer formation and secretion of both half-molecules and dimeric fibrinogen (Fig. 3, A and B, lane 3). Further deletion of another 8 amino acids (Bβ1–80), which includes deletion of a pair of cysteines flanking the amino-terminal side of the coiled-coil region, completely eliminated the formation of dimeric fibrinogen, and only half-molecules were assembled and secreted (Fig. 3, A and B, lane 4). In all cases small amounts of Aαγ complexes were secreted. The radioactive protein at the top of the gels, marked by arrows, is a non-fibrinogen contaminant expressed by COS cells (19).

The half-molecule, which contains Bβ1–80, migrated more slowly than expected in comparison with the half-molecule that
contains BβΔ1–72. However, two-dimensional gel electrophoresis confirms that they are both half-molecules and suggests that the differences in mobilities may be due to differences in conformation (Fig. 4).

A previous study showed that disruption of the disulfide rings that flank the carboxyl-terminal side of the coiled-coil region, by substituting cysteine with serine residues, allowed dimeric fibrinogen to be formed but prevented secretion. To further examine the role of the carboxyl-terminal disulfide rings in assembly and secretion, a Bβ chain mutant (BβD1–192), which contains the carboxyl-terminal pair of cysteines at positions 193 and 196 but does not contain the amino-terminal domain or the coiled-coil region, was expressed. This deletion mutant did not assemble with normal Aα and γ chains, and no half-molecules or dimeric fibrinogen were detected on SDS-PAGE under nonreduced conditions either intracellularly (data not shown) or in the culture medium (Fig. 3A, lane 5). However, a small amount of Aαγ complex and free γ chains were secreted, and Aα and γ chains were not detected on reduction (Fig. 3B, lane 5). The free γ chain is not noted in Fig. 3A, lane 5, because in order to separate the nonreduced fibrinogen complexes, a 5% gel was used, and the free γ chain migrated out of the system.

Analysis of the Secreted Bβ Mutants by Two-dimensional Gel Electrophoresis—To further characterize the fibrinogen complexes secreted, the radioactive proteins were separated in the first dimension by nonreduced SDS-PAGE and in a second
Expression of normal $\alpha$, $\beta$, and $\gamma$ resulted in the secretion of dimeric fibrinogen. Detectable, only after overexposure of the film, were small amounts of half-molecule, $\alpha\gamma$, and $\gamma\gamma$ complexes. The principal secreted product, dimeric fibrinogen, yielded, as expected, $\alpha\beta$, $\beta\gamma$, and $\gamma\gamma$ chains on reduction (Fig. 4, top left panel). The area identified as $\alpha\gamma\gamma$ complex contained more radioactivity in the $\alpha\gamma$ than in the $\gamma\gamma$ chain, and therefore it is possible that this area may also contain a mixture of $\alpha\alpha\alpha\beta\gamma\gamma$ dimer and small amounts of a $\gamma$ homopolymer.

Expression of $\alpha\alpha$, $\beta\Delta 1-72$, and $\gamma$ led to secretion of mutant dimeric fibrinogen, mutant half-molecule, and $\alpha\alpha\gamma$ complex. In both the mutant dimeric fibrinogen and mutant half-molecule, the $\beta\Delta 1-72$ migrated faster in the second dimension than $\gamma$ chain (Fig. 4, top right panel).

As shown in Fig. 3A, lane 4, expression of $\alpha\alpha$, $\beta\Delta 1-80$, and $\gamma$ led to inhibition of dimer formation and secretion of half-molecules. The mutant half-molecule was characterized by its appropriate mobility in the first dimension and that it is composed of $\alpha\alpha$, mutant $\beta\beta$, and $\gamma$ chains (Fig. 4, bottom left panel). Another fibrinogen complex migrated slightly faster than the half-molecule in the first dimension, and because of its size, chain composition, and variability in different experiments, it was tentatively identified as degraded half-molecule. Small amounts of mutant fibrinogen chains were also noted in the second dimension in a higher molecular weight complex, suggesting that larger complexes composed of $\alpha\alpha$ and mutant $\beta\beta\Delta 1-80$ chains may also occur.

Deletion of Amino-terminal Domains of $\alpha$, $\beta$, and $\gamma$ Chains—To determine the effect of amino-terminal amino acid residues of $\alpha$, $\beta$, and $\gamma$ chains in dimer formation, mutants were created, equivalent to $\beta\Delta 1-72$, which deleted the amino-terminal amino acid residues upstream from the disulfide rings, which flank the amino-terminal side of the coiled-coil region. COS cells were co-transfected with a mutant chain and the two corresponding normal chains, and fibrinogen assembly and secretion were determined. In contrast to $\beta\Delta 1-72$, (Fig. 5, lane 3) in which both half-molecules and dimeric fibrinogen were secreted, removal of the first 41 amino acids of $\alpha\alpha$ chain ($\alpha\alpha\Delta 1-41$) did not affect the assembly and secretion of dimeric fibrinogen (Fig. 5, lane 2). Dimeric fibrinogen was secreted into the culture medium. Similarly, deletion of the first 15 amino acids of $\gamma$ chain ($\gamma\Delta 1-15$), did not interrupt dimer formation, and dimeric fibrinogen was secreted (Fig. 5, lane 4). However, COS cells co-expressing mutants $\alpha\alpha\Delta 1-41$, $\gamma\Delta 1-15$, and normal $\beta\beta$ chains, only assembled and secreted half-molecules and...
Amino termini of Aα and γ chains participate in dimer assembly. COS cells, co-transfected with normal or mutant fibrinogen chain cDNAs were incubated with L-[^35S]methionine for 2 h. Fibrinogen complexes were immunoprecipitated, separated on SDS-PAGE under nonreduced conditions, and detected by autoradiography. Panel A shows intracellular fibrinogen chains on 7.5% SDS-PAGE. Panel B shows secreted fibrinogen chains on 5% SDS-PAGE. Lane 1, normal Aα, Bβ, and γ; lane 2, AαΔ1–41, Bβ, and γ; lane 3, Aα, BβΔ1–72, and γ; lane 4, Aα, Bβ, and γΔ1–15; lane 5, AαΔ1–41, Bβ, and γΔ1–15. Lanes 1, 2, and 3 are from the same experiment, and lanes 4 and 5 were analyzed at different times. Mb+, normal fibrinogen; mFb, mutant fibrinogen; mHβFb, mutant half-molecule of fibrinogen.

did not form dimeric fibrinogen (Fig. 5, lane 5). The half-molecule assembled in these conditions was further characterized by two-dimensional SDS-PAGE analysis (Fig. 4, bottom right panel). It had the appropriate mobility in the first dimension and yielded mutant Aα and γ chains and normal Bβ on the second dimension. This is in agreement with previous studies in which amino-terminal cysteine residues were changed to serine (19, 30). AαΔ1–41 and γΔ1–15 do not contain the cysteine residues (Aα Cys28, Aα Cys36, and γ Cys6 and Cys8), which link, by disulfide bonds, the two half-molecules of fibrinogen.

Noncovalent Interactions in Dimer Assembly: Comparison of Amino-terminal Deletion Mutants with Corresponding Cysteine to Serine Substitutions—Previous studies indicated that the half-molecules of fibrinogen are linked by three symmetrical disulfide bonds between adjacent Aα and γ chains (Aα Cys28–Aα Cys28, γ Cys8–Cys8, and γ Cys6–Cys6) and probably between Aα Cys36 of one half-molecule and Bβ Cys55 of the other half-molecule. This conclusion was reached because substituting cysteine residues with serine at Aα 28 and γ 8 and 9 did not abolish dimer formation, but if the cysteines at Aα 36 or Bβ 65 were also changed to serine, then half-molecules and not dimers were mostly formed (19, 30). There was, however, a difference depending on whether the cysteines at Aα 36 or Bβ 65 were substituted. When Aα Cys36 was changed to serine some dimer formation occurred, and when Bβ Cys55 was changed to serine very few dimers were formed (30). This could be interpreted as either due to a rearrangement of disulfide interactions or the result of other noncovalent interactions between the half-molecules. To determine if, in addition to disulfide linkages, noncovalent interactions also occur between the amino-terminal domains of the two half-molecules, we compared deletion mutants with substitution mutants, in which the participating cysteine residues were substituted with serine.

As described earlier, truncation of the first 41 amino acids of the Aα chain, which eliminates Aα cysteines 28 and 36 (Fig. 6A, lane 2), or truncation of the first 15 amino acids of γ chain, which eliminates cysteines 8 and 9 (Fig. 6A, lane 6), did not prevent dimer formation and secretion if these mutants were co-expressed with the two other normal chains. Similar results were obtained by substituting serine for cysteine residues at Aα Cys28 and Cys36 (Fig. 6, lane 3) and at γ Cys8 and Cys9 (γ8s,9s, Fig. 6A, lane 7). However, co-expressing AαΔ1–41, γΔ1–15, and BβΔ1–72, which removes all of the amino-terminal cysteine residues (Aα Cys28 and Cys36, γ Cys8 and Cys9, and Bβ Cys55) that are thought to hold the half-molecules together, resulted in the formation and secretion of only half-molecules (Fig. 6A, lane 14). It should be noted (Fig. 6B, lanes 7, 11, 13, and 15) that expression of γ8s,9s leads to the addition
of an extra N-linked sugar and results in the mutant γ chain migrating close to normal Bβ chains (18).

To determine whether the first 41 amino acids of the Aα chain and the first 15 amino acids of γ chain interact noncovalently and assist dimeric fibrinogen assembly, a series of co-transfections were performed. COS cells co-expressing Aα, Bβ, and γ8s,9s (Fig. 6A, lane 11) secreted a mixture of half-molecules and dimeric fibrinogen, indicating that dimer formation was affected but not completely abolished. Similar results were obtained with AαD1–41, normal Bβ, and γ8s,9s (Fig. 6A, lane 16). By contrast COS cells co-expressing AαD1–41, normal Bβ, and γD1–15 (Fig. 6A, lane 10) predominantly secreted half-molecules and very little dimeric fibrinogen. The chain composition of the mutant half-molecule is shown in two-dimensional gels in Fig. 4. Also, co-expression of Aα28s,36s, normal Bβ, and γD1–15 (Fig. 6A, lane 17) led to the formation and secretion of half-molecules, with very little dimeric fibrinogen. These results indicate that noncovalent interactions at the amino-terminal domain of the γ chain, besides covalent interactions of cysteines, may also be involved in the formation of the dimeric complexes.

Truncation of the first 72 amino acids of the Bβ chain also inhibited dimer formation, and both half-molecules and dimers were secreted (Fig. 6A, lane 4). This region of the Bβ chain contains a cysteine at position 65 that is thought to be linked to the cysteine at Aα 36 of the other half-molecule of fibrinogen. However, only substituting Bβ cysteine 65 to serine did not affect dimer formation and secretion (Fig. 6A, lane 5). This again suggests that amino acid residues within Bβ 1–72 other than the cysteine residues are involved in dimer formation by noncovalent interactions.

On reduction, the secreted fibrinogen complexes in all cases only contained the expected mutant or normal fibrinogen chains (Fig. 6B).

**DISCUSSION**

The two half-molecules of fibrinogen are linked by amino-terminal disulfide bonds. Earlier studies indicated that the three symmetrical disulfides, between adjacent cysteines at Aα 28 and γ 8 and 9 were the principal disulfide interactions holding the two half-molecules together. However, substituting these cysteine residues with serine did not abolish dimer formation in transfected COS cells (18). It was later shown that if, in addition, the cysteine residues at Aα 36 or Bβ 65 were also substituted with serine, then dimer formation was inhibited. This led to the suggestion that, in addition to the symmetrical disulfide bonds between adjacent Aα and γ chains, cysteine Aα 36 of one half-molecule is linked to cysteine Bβ 65 of the other half-molecule (19, 30). However, these amino-terminal disulfide bonds may not be the only interactions holding the two half-molecules. For example, substitution of Bβ Cys 65 with serine did not affect dimer formation, but deleting the first 72 amino-terminal amino acids inhibited dimer formation, and equal amounts of half-molecule and dimer were secreted. This suggested the involvement of some of the amino-terminal amino acid residues of the Bβ chain, other than cysteine, in dimer formation.

In this report we test other Bβ deletion mutants and also amino-terminal Aα and γ deletion mutants and demonstrate that, in addition to the cysteine at position 65, sequences within the first 72 amino acid residues of Bβ participate in dimer formation as do amino-terminal residues in the Aα and γ chains. The results further suggest that a linear amino acid sequence within the Bβ amino-terminal region may not be responsible for linking the two half-molecules, since BβΔ1–8 had little or no effect on dimer formation; BβΔ9–72 slightly inhibited dimer formation, but BβΔ1–72 significantly interrupted dimer formation.

BβΔ9–72 is the same as a naturally occurring mutant fibrinogen, termed Fibrinogen New York 1, which was identified as a heterozygous congenital dysfibrinogenemia with thrombotic tendency. In the patient, Fibrinogen New York 1 was present, in plasma, as a dimer. In the recombinant system small amounts of half-molecules are also secreted when BβΔ9–72 was co-expressed with normal Aα and γ chains. If some half-molecules were present in the plasma of Fibrinogen New York, they could remain undetected because plasma fibrinogen was not analyzed in nonreduced SDS-PAGE gels. Alternatively, in vivo, the half-molecule may not be secreted by hepatocytes or may be quickly cleared from the circulation.

Clearly the major link between the two half-molecules is the amino-terminal disulfide bond (19, 30). However, our current results indicate that noncovalent interactions in all three chains also participate. For example, COS cells co-expressing Aα28s,36s, γ8s,9s, and normal Bβ chains are capable of assembling and secreting equal amounts of dimeric fibrinogen and half-molecules. By contrast, cells that co-expressed AαD1–41, γD1–15, and normal Bβ chains only assembled and secreted half-molecules. This indicates that besides cysteines at Aα 28 and 36 and γ 8 and 9, noncovalent interactions of other residues in the regions of AαD1–41 and γD1–15 participate in dimer formation. Further analysis showed that COS cells co-expressing AαΔ1–15, γ8s,9, and normal Bβ chains secreted both dimer and half-molecules, while COS cells co-expressing Aα28s,36s, γD1–15, and normal Bβ chains only secreted half-molecules, indicating that noncovalent interactions of γD1–15 residues may play a more important role than that of Aα chain in holding the two half-molecules together.

A structural feature of the three fibrinogen chains is that each chain contains about 111 hydrophobic amino acids, which intertwine to form an α-helical coiled-coil region. The coiled-coil region is flanked by a pair of cysteine (Cys-X-X-Cys) residues, which form interchain disulfide bridges, termed disulfide rings. Theoretical considerations suggested that the coiled-coil domain and the disulfide ring may play a key role in fibrinogen assembly (4). Our early studies demonstrated that the first 207 amino acids of the Bβ chain, which contains the amino-terminal residues and an intact coiled-coil domain with disulfide rings, can form a dimeric structure when co-expressed with normal Aα and γ chains (17). The present data show that removal of the first 72 amino acids of the Bβ chain results in assembly of the three chains but that dimer formation is inhibited. This indicates that amino acid residues 73–207 of the Bβ chain are sufficient for assembly of half-molecules, while a subset of amino acid residues 1–72 of Bβ chain are necessary for dimer formation. Our data also demonstrate that deletion of an amino-terminal segment that contains the cysteine residues that are part of the disulfide rings completely eliminates dimer formation. This is consistent with our earlier studies, which showed that disruption of the disulfide rings by substituting cysteine residues with serine abolished dimer formation.

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