Fibrinogen Paris I contains a mutant γ chain that is longer than the normal chain, resulting in altered fibrin polymerization and cross-linking. Because these functions involve the carboxy-terminal region of the γ chain, we decided to determine whether fibrinogen Paris I or the isolated Paris I γ chain supports normal ADP-induced platelet aggregation, a function that requires the ultimate 12 residues of the normal γ chain (400 through 411). Aggregation of ADP-stimulated normal platelets was defective with fibrinogen Paris I and markedly depressed with the γ Paris I chain. These findings prompted us to characterize the carboxy-terminal structure of the region of the γ Paris I chain responsible for this activity. The carboxy-terminal cyanogen bromide (CNBr) peptide of the normal γ chain (385 through 411) or that from γ Paris I was isolated by differential adsorption to triethylene-tetramine resin or by reverse-phase high-performance liquid chromatography (HPLC). The CNBr peptide from the Paris I γ chain was identical to that of the normal γ chain in its retention time on HPLC, its amino acid composition, and its sequence. Thus, the primary structure of the γ Paris I chain from residue 384 through 411 is normal, indicating that a peptide insertion has occurred upstream from residue 384, resulting in an impairment of those physiologic functions attributable to the carboxy-terminal end of the γ chain from position 384 (ie, cross-linking, ADP-induced platelet aggregation, and at least a portion of the γ chain polymerization site). These observations demonstrate that the γ chain platelet recognition site in the fibrinogen molecule is necessary but not alone sufficient to support normal ADP-induced platelet aggregation. There appears to be an additional requirement for normal conformation of the γ chain or availability of its terminal sequence during the interaction of fibrinogen with platelets.

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Materials

Guandine hydrochloride ultrapure was purchased from Schwarz Mann, division of Beckton Dickinson (Orangeburg, NY), reagents used for polycrylamide gel electrophoresis (PAGE), cyanogen bromide (CNBr), and iodoadic acid from Eastman Kodak (Rochester, NY); Tris aminomethane (Tris), dithiothreitol (DTT), sodium dodecyl sulfate (NaDodSO4), N-ethylmorpholine (NEM), Hepes, apyrase grade V, prostaglandin E, (PGE), bovine carboxypeptidase A (DFP treated) and standard L-amino acids were purchased from Sigma Chemical (St Louis); bovine serum albumin (BSA) fraction V from Miles (Elk Hart, IN); aprotinin from Choay (Paris), ADP from Stago (Asnières, France); Sephadex G 100 from Pharmacia (Uppsala, Sweden); carboxymethylcellulose CM 23 from Whatman (Maidstone, England); Biorex RG 501-X,8,DC-6A resin from Durrum (Palo Alto, CA), aquapore RP-300 from Brownlee (Santa Clara, CA); acetonitrile HPLC grade from Fisons (Loworough, England); dimethylaminobenzene isothiocyanate (DABITC) and phenylisothiocyanate (PITC) from BDH (Poole, England); dansyl-chloride, aminopropylstereen resin, triethyl-enetetramine resin, dimethylformamide, triethylamine from Pierce Chemical (Rockford, IL); and polyamide sheets from Schleicher and Schuell (Dassel, FRG). When unspecified, chemicals were reagent grade or better.

Proteins and Peptide Isolation

Blood from the propositus and normal donors was collected in 0.13 mol/L of sodium citrate containing 560 KIU/mL aprotinin (1 vol of anticoagulant/9 vol of blood).

Fibrinogen. Fibrinogen was isolated from plasma as previously described.\(^\text{25}\) Fibrinogen concentrations were determined from absorbance reading at 280 nm assuming an extinction coefficient of 1.52 mL/mg/cm.\(^\text{36}\)

Normal fibrinogen \(\gamma\) chain. Normal fibrinogen \(\gamma\) chain was isolated as follows: after dialysis against 50 mmol/L Tris, 0.1 mol/L of NaCl, pH 7.4, fibrinogen was reduced and S-carboxymethylated.\(^\text{27}\) The Aa, Bf, and \(\gamma\) chains were separated by chromatography on CM 23 cellulose\(^\text{28}\) using deionized urea\(^\text{29}\) for the gradient solution consisting of 8 mol/L of urea, 0.1 mol/L of acetic acid, buffered at pH 4.8 to 5.8 with Tris. The elution profile was as previously described.\(^\text{30}\) Purity of the normal \(\gamma\) chain was ascertained by NaDodSO\(_4\) PAGE analysis.\(^\text{31}\)

\(\gamma\) Paris I chain. The inability of \(\gamma\) Paris I chain to cross-link in the presence of factor XIIa was turned to an advantage for its purification, as described by Stathakis and colleagues.\(^\text{32}\) In brief, cross-linked fibrin Paris I was prepared by recalcification of plasma; all the normal \(\gamma\) chains in the clot underwent covalent cross-linking to \(\gamma\)-dimers, whereas \(\gamma\) Paris I chains did not. The fibrin clot was then reduced, S-carboxymethylated,\(^\text{27}\) and sieved on Sephadex G 100 (column 2.5 \times 90 cm, 9.4 mL/h) equilibrated in a freshly deionized 8 mol/L of urea, 0.1 mol/L of CH\(_3\)COOH solution, buffered at pH 5.0 with Tris. Further purification of the \(\gamma\) Paris I chain was achieved by chromatography on CM 23 cellulose, as described above.

CNBr peptides from the normal and Paris I S-carboxymethyl \(\gamma\) chains. CNBr peptides were obtained by CNBr cleavage of the methionyl bonds, in 70% vol/vol formic acid, using CNBr in a 120-fold molar excess with respect to the methionyl residues. After 24 hours at room temperature, the digests were diluted 1:10 with water and lyophilized. The carboxy-terminal CNBr peptide was isolated by the method of Horn,\(^\text{34}\) as recently applied to the preparation of the carboxyl-terminal CNBr fragment of rat fibrinogen gamma chains.\(^\text{35}\) In brief, TFA (200 \(\mu\)L) was added to the CNBr digests (200 \(\mu\)g). After incubation for 1 hour at 22°C, the solutions were dried under a stream of nitrogen. Dimethylformamide (0.5 mL) was then added to the residues, followed by a slurry of 100 mg of aminopropylstyrene and triethylammonium resin. After the addition of triethylamine (100 \(\mu\)L), followed by 12 hours of stirring at 45°C, the suspensions were centrifuged and the supernatant containing the carboxy-terminal peptide was freeze-dried. The carboxy-terminal CNBr peptide was also purified by HPLC on an aquapore RP-300 column (0.46 \times 25 cm). The gradient used was acetonitrile in 50, 15, and 60 minutes respectively, at a flow rate of 1.5 mL/min. The absorbance was monitored at 220 nm.

Amino Acid and Sequence Analysis

Amino acid analyses were performed by subjecting the peptides to hydrolysis in distilled 6 N HCl for 22 hours at 110°C under vacuum. Amino acids were identified by chromatography on a DC-6A column using an automatic amino acid analyzer (Liquimat 2, Labotron, Kontron Instruments, Zurich); amino acid detection was achieved with O-phthalaldehyde.\(^\text{36}\) The carboxy-terminal residue was identified as follows: the \(\gamma\) Paris I chain (200 \(\mu\)g) was dissolved in a 0.1 mol/L N-ethylmorpholine acetate buffer, pH 8.1, and 2 \(\mu\)g of carboxypeptidase A (previously diaлизed against 10,000 vol of buffer) was added. After 1 hour at 37°C, the solution was freeze-dried, dissolved in 50 \(\mu\)L of 0.5 mol/L of Na bicarbonate buffer, pH 9.8, containing 1% (wt/vol) NaDodSO\(_4\) subjected to dansylation, and the carboxy-terminal amino acid was identified by both TLC\(^\text{37}\) and HPLC.\(^\text{38}\)

Amino-terminal amino acid sequencing was performed by the manual liquid-phase DABITC/PITC procedure\(^\text{39}\) or alternatively on an automatic gas-phase microsequencer from Applied Biosystems (Foster City, CA). The PTH derivatives were analyzed by HPLC.\(^\text{40}\)

Platelet Aggregation

Platelet-rich plasma (PRP) was prepared from human blood collected into acid-citrate-dextrose (ACD-A) (6 vol of blood/1 vol of anticoagulant), freshly drawn from healthy volunteers who had abstained from taking aspirin or other medication during the 10 preceding days. ACD-A (10% vol/vol), apyrase (25 \(\mu\)g/mL), and PGE\(_1\) (100 nmol/mL) were added to PRP. The platelet pellet obtained by centrifugation for 15 minutes at 1,500 g at 20°C was resuspended in a washing buffer\(^\text{41}\) consisting of 36 mmol/L citric acid, 5 mmol/L of glucose, 5 mmol/L of KCl, 2 mmol/L of CaCl\(_2\), 1 mmol/L of MgCl\(_2\), 103 mmol/L of NaCl, pH 6.5, 25 \(\mu\)g/mL of apyrase, 100 nmol/L of PGE\(_1\). Platelets were separated by centrifugation for 20 minutes at 1,500 g through a stepwise discontinuous gradient\(^\text{42}\) consisting of 8%, 10%, 13%, 20%, and 40% albumin in the washing buffer. The platelet fraction was collected onto the 40% albumin cushion, washed twice in the washing buffer, and then resuspended in a reaction buffer consisting of 137 mmol/L of NaCl, 2 mmol/L of KCl, 12 mmol/L of NaHCO\(_3\), 0.3 mmol/L of NaH\(_2\)PO\(_4\), 2 mmol/L of CaCl\(_2\), 1 mmol/L of MgCl\(_2\), 5 mmol/L of glucose, 5 mmol/L of Hepes, pH 7.4, 0.35% BSA. The platelet count was adjusted to 3 \times 10\(^8\)/mL, and the platelets were used within 2 to 3 hours after blood collection.

Platelet aggregation was studied by measuring light transmission through a suspension of platelets stirred at 1,100 rpm in a Coulter aggregometer (Coulter Electronics, Hialeah, FL). Purified fibrinogen or \(\gamma\) chains (60 \(\mu\)L) dissolved in 50 mmol/L of NH\(_4\)HCO\(_3\), pH 7.8 (0.25 to 4 mg/mL) were added to 300 \(\mu\)L of PRP. The platelet aggregation was induced by the addition of 40 \(\mu\)L of ADP (final concentration 5 \(\mu\)mol/L). Platelets prepared as described above did not respond to ADP without added fibrinogen.

RESULTS

Purification of \(\gamma\) Paris I Chains

When reduced and S-carboxymethylated cross-linked fibrin Paris I was subjected to gel filtration on Sephadex G 100, the chromatogram consisted of a single broad peak, with only a partial purification of the \(\gamma\) Paris I chain. Further purification on CM 23 cellulose yielded pure \(\gamma\) Paris I chain, which exhibited a 3,000-dalton difference in molecular mass when compared with normal \(\gamma\) chain by NaDodSO\(_4\) PAGE (Fig 1).

ADP-Induced Platelet Aggregation in the Presence of Fibrinogen Paris I or \(\gamma\) Paris I Chain

In the presence of normal fibrinogen, aggregation of normal platelets is readily induced by the addition of ADP (Fig 2). When normal fibrinogen was replaced by equal amounts of fibrinogen Paris I, the rate and extent of platelet aggregation were reduced by approximately twofold, and the initial aggregation was followed by disaggregation.

As previously reported by Hawiger et al,\(^\text{15}\) isolated normal \(\gamma\) chains support ADP-induced platelet aggregation (Fig 3,
Fig 1. Gel electrophoretic analysis of γ Paris I chain. Samples of purified normal fibrinogen (lane 1), cross-linked fibrin Paris I (lane 2) and γ Paris I chain isolated on CM23-cellulose (lane 3) were reduced and subjected to electrophoresis in 10% acrylamide, 0.1% NaDodSO₄.

A linear relationship between the aggregation rate and the γ chain concentration was observed, at concentrations ranging from 0.75 to 12 μmol/L, with complete aggregation observed at 12 μmol/L of γ chain. Both the rate and extent of ADP-induced platelet aggregation were markedly depressed in the presence of purified γ Paris I chains (Fig 3, curve 1). A 30% reduction was observed in the ultimate extent of aggregation (Fig 3, curve 3) as compared with a comparable dilution of normal γ chain with buffer alone (Fig 3, curve 4). No difference was detected when a 1:1 ratio was used (not shown).

The effect of purified γ Paris I chain toward platelet aggregation in the presence of normal γ chain was studied by performing mixing experiments. When a mixture of γ Paris I and normal γ chains in a ratio of 3:1 was used, a 30% reduction was observed in the ultimate extent of aggregation (Fig 3, curve 3) as compared with a comparable dilution of normal γ chain with buffer alone (Fig 3, curve 4). No difference was detected when a 1:1 ratio was used (not shown).

Fig 2. Effect of normal fibrinogen and fibrinogen Paris I on ADP-induced platelet aggregation. Normal (curve 1) or Paris I (curve 2) fibrinogen was added to 300 μL of washed platelets prior to ADP stimulation. The final concentrations were: platelets 3 x 10⁵/mL, fibrinogen 1.84 μmol/L, and ADP 5 μmol/L. T, light transmission.

Fig 3. Effect of normal γ chains and γ Paris I chains on ADP-induced platelet aggregation. Purified γ chains were added to 300 μL of washed platelets (3 x 10⁵/mL) prior to ADP (5 μmol/L) stimulation. (1) Normal γ chain 4.5 μmol/L. (2) γ Paris I chain 4.5 μmol/L. (3) Mixture of normal (1.12 μmol/L plus Paris I (3.38 μmol/L) γ chains. (4) Normal γ chains 1.12 μmol/L. (5) Buffer. T, light transmission.

Isolation and Analysis of CNBr Carboxy-Terminal Peptides From Normal and Paris I γ Chains

The carboxy-terminal CNBr peptide was isolated from both normal and Paris I γ chains digests, as described by Horn. Internal fragments containing lactonized homoserine residues (HSe) bound covalently to the resin, whereas the carboxy-terminal CNBr peptide was recovered in the supernatant. Manual Edman degradation of the unbound material, in both cases, identified a Lys-Ile-Ile-Pro amino-terminal sequence, corresponding to residues 385 through 388 in the γ chain (Table 1). The carboxy-terminal residue released on carboxypeptidase A treatment of these two peptides was Val in both cases, as was the carboxy-terminal residue of the intact Paris I or normal γ chain.

On reverse-phase HPLC, the normal and Paris I carboxy-terminal CNBr peptides exhibited an identical retention time. The amino acid composition of the two peptides so purified was very similar (Table 2). Amino-terminal sequence analysis by the manual procedure extended our previous identification for five residues for the normal peptide and for 11 residues in the case of fibrinogen Paris I. Finally, with the use of a gas-phase microsequencer, we obtained the entire amino acid sequence of the 27-residue Paris I peptide. Indeed, it corresponds exactly to the normal sequence of the γ chain extending from residue 385 to the carboxy-terminal Val 411 (Table 1). Three additional degradation cycles gave no evidence for any liberated PTH amino acids beyond Val 411.

DISCUSSION

Human fibrinogen γ chain is involved in several functions, all of which require the participation of its carboxy-terminal region. A complementary polymerization site is located within segment 374 through 396 and the cross-linking sites between positions 398 and 406. The 397 through 405 segment participates in the interaction with the staphylococcal clumping receptor, whereas the continuous sequence consisting of the ultimate carboxy-terminal amino acids 400 through 411 of the γ chain constitutes the platelet membrane recognition site. Fibrinogen Paris I is characterized by the
coexistence of normal and elongated \( \gamma \) chains.\(^{21,22}\) This report demonstrates that, in addition to the previously described defects in polymerization,\(^{46}\) cross-linking\(^{21,22}\) and plasmic degradation,\(^{23}\) both the rate and extent of ADP-induced platelet aggregation are depressed in the presence of fibrinogen Paris I. Furthermore, the isolated \( \gamma \) Paris I chain is unable to support ADP-induced platelet aggregation, even though its terminal sequence is normal from position 384 and includes residues 397 through 411, which account for this activity in normal \( \gamma \) chains.

The functional defect of the carboxy-terminal part of Paris I \( \gamma \) chain is therefore not related to an abnormality in the primary structure of the region extending from residue 384 through residue 411, and the elongation of the \( \gamma \) Paris I chain is due to an internal insertion of peptidic material upstream from Met 384. As suggested by \( \text{NaDodSO}_4 \) PAGE,\(^{21}\) this insertion consists of \(-22\) residues. To our knowledge, this constitutes the largest internal insertion observed to date in human protein mutants. Most of the other elongated variants result either from a frameshift mutation in the vicinity of the termination codon or from a mutation of this codon itself and therefore have an abnormal carboxy-terminal extremity.

The \( \gamma \) Paris I chain may be unable to bind to the platelet receptor because of a conformational change that results in masking of its carboxy-terminal region. Alternatively, the binding of \( \gamma \) Paris I chains to stimulated platelets, although occurring through the normal carboxy-terminal recognition site, may result in ineffective bridging between adjacent platelets. We favor this second possibility because receptor occupancy by elongated and nonfunctional \( \gamma \) chains could account for an inhibitory effect and explain the disaggregation phenomenon that occurs with platelet aggregated with fibrinogen Paris I. Defective binding to platelets has also been described for the extended \( \gamma \) variant of normal human fibrinogen,\(^{48}\) but in this case, deletion of the terminal residues 408 through 411 of the specific platelet recognition site probably accounts for this behavior.\(^{48}\)

The \( \gamma \) Paris I chain is absent (or present in markedly reduced amounts) from the Paris I subject's platelet fibrinogen.\(^{49}\) In addition, the plasma fibrinogen \( \gamma \) variant is not found in normal platelet fibrinogen.\(^{35,50}\) These observations raise the question of whether the ability of the \( \gamma \) chain to be packaged in platelet \( \alpha \)-granules is dependent on its ability to bind to a membrane receptor for the \( \gamma \) chain.

In conclusion, study of the reactivity of fibrinogen Paris I and \( \gamma \) Paris I chains in platelet aggregation has provided a unique opportunity to demonstrate that the presence of the \( \gamma \) chain platelet recognition site in the fibrinogen molecule is necessary but not alone sufficient to support normal ADP-induced platelet aggregation. That is, there appears to be an absolute requirement for normal conformation of the \( \gamma \) chain or availability of its carboxy-terminal sequence during the interaction of fibrinogen with platelets.

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