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QUARTERLY

The amino acids that constitute sequence γ 268-282 of fibrinogen are not involved in fibrin monomer polymerization*

Anna Janiak[1], Tomasz Plucinski[2], Gotfryd Kupryszewski[2] and Czesław S. Cierniewski[1]**

Department of Biophysics[1], Medical University in Łódź, and Faculty of Chemistry[2], University of Gdańsk, Poland

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Congenitally abnormal fibrinogens with impaired fibrin monomer polymerization have been described to contain single amino-acid substitutions localized in certain positions of the $\gamma_{275-330}$ peptide region. To evaluate the role of the amino-acid sequence in the vicinity of Arg^{275} in fibrin monomer polymerization, the peptide fragment corresponding to $\gamma_{268-282}$ was synthesized and used to obtain peptide-specific antibodies. These antibodies, when purified immunochemically on the immobilized peptide, bound to the intact fibrinogen and fibrin monomers with the same binding affinity. However, they did not recognize the $\gamma_{268-282}$ epitopes on the denatured and reduced fibrinogen molecules. The lack of influence of antipeptide antibodies on fibrin monomer polymerization indicates that the $\gamma_{268-282}$ peptide is not directly involved in the structure of the polymerization site in the D domain of fibrinogen. It is suggested that substitution of Arg^{275} either by His or Cys in abnormal fibrinogens results probably in conformational changes which disturb a proper orientation of the polymerization site and reduce its expression.

The COOH-terminal region of the γ chain of fibrinogen has been shown to participate in various functions of fibrinogen, including fibrin monomer polymerization [1 - 4]. This region is a part of the D domain of fibrinogen molecule and was described to contain a polymerization site [5 - 9], localized tentatively within the sequence 275 - 405 [5]. However, specific amino-acid residues involved in the structure of this polymerization site are still unknown. The significance of the NH_2-terminal part of this segment, i.e. $\gamma_{275-330}$, is indicated by the existence of a number of abnormal fibrinogens in which single amino acid substitutions affecting fibrin assembly were identified. These natural mutations provide some information about amino-acid residues that may directly or indirectly participate in the structure and expression of the polymerization sites. A number of congenitally abnormal fibrinogens were found to contain Arg^{275} substituted by His (10 - 12) or Cys (5, 13, 14). Such a single substitution perturbed the structure required for fibrin monomer polymerization, specifically that assigned to the COOH-terminal D domain of fibrinogen. In order to evaluate the role of Arg^{275} and cognate sequences in fibrin monomer polymerization, in the work reported here we synthesized the peptide segment $\gamma_{268-282}$ and used it to produce antibodies. Antipeptide antibodies and the synthetic peptide had no effect on fibrin monomer polymeri-

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**To whom correspondence should be addressed: Department of Biophysics, Medical University in Łódź, 90–131 Łódź, Lindleya 3, Poland

zation indicating that this sequence does not form part of the polymerization site present in the D domain of fibrinogen.

MATERIALS AND METHODS

Materials. All chemicals and reagents were from the sources indicated: CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals (Uppsala, Sweden), 4-chloro-1-naphthol and nitrocellulose membranes, Bio-Rad; horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin, Polclone (Lask, Poland), acetonitrile and trifluoroacetic acid, J.T. Baker (Holland).

Fibrinogen purification. Human fibrinogen was isolated from fresh blood plasma by differential ethanol precipitation [15] and was additionally purified by ammonium sulfate precipitation at 26% saturation. In addition, fibrinogen preparations were freed from plasminogen and fibronectin contaminants by chromatography on the lysine-Sepharose and gelatin-Sepharose columns, respectively. Polyacrylamide gel electrophoresis of the reduced fibrinogen in the presence of sodium dodecyl sulfate demonstrated purity of the preparations, as only intact Aα, Bβ, and γ chains were present.

Peptide synthesis. The peptide GPEADKYRLTYAYFA corresponding to $\gamma_{268-282}$ was synthesized by the solid-phase method [16] using chloromethylated resin (substitution 0.67 meq/g). The C-terminal amino acid (Boc-Ala) was coupled to the resin by the procedure of Horiki *et al.* [17]. Then a portion of 1.0 g of Boc-Ala-OCH_2-resin was used for peptide synthesis carried out with stirring. Reversible protection of the α-amino groups was obtained with t-butyloxycarbonyl (Boc) group and, in addition, the following side-chain protecting groups were used: Glu(OBzl), Asp(OBzl), Lys(Z-Cl), Arg(Tos), Thr(OBzl), Tyr(OBzl). Deblockings were performed with 40% trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2) in the presence of 1% anisole or dimethylsulfide (DMS) and neutralization with 10% triethylamine in CH_2Cl_2 (twice). Double couplings in CH_2Cl_2 were achieved using 2.0 M dicyclohexylcarbodiimide in CH_2Cl_2. The protected amino acid was used at a two-fold excess in relation to the original substitution of Boc-Ala-OCH_2-resin. Coupling efficiency was monitored according to the test of Kaiser *et al.* [18]. The peptide was cleaved from the resin by the HF procedure [19]. Crude product (0.78 g) was purified on Sephadex G-25 column (3.8 cm × 110 cm) using 50% acetic acid as an eluent and then on LH-20 column (3.8 cm × 110 cm) with 20% acetic acid as an eluent, yielding 100 mg of the product. The peptide was finally purified by preparative HPLC using two connected preparative columns (Vydac C_{18} silica, 30 μm particle size, 35 mm × 250 mm). A solvent system of 0.2 M triethylammonium phosphate in 13% isopropanol - 87% H_2O (pH 2.0) was used under isocratic conditions. The purity of the peptide was checked by reverse phase HPLC (Ultrasphere ODS RP_{18}, 4 mm × 150 mm) in a linear gradient from 30% to 50% of acetonitrile in 0.1% TFA and amino acid composition analysis which showed the presence of Gly – 1.00 (1), Pro – 0.89 (1), Glu – 1.11 (1), Ala – 2.78 (3), Asp – 1.16 (1), Lys – 1.15 (1), Tyr – 2.81 (3), Arg – 1.10 (1), Leu – 0.94 (1), Thr – 0.90 (1), and Phe – 1.08 (1).

Antipeptide antibodies. The peptide was conjugated with bovine serum albumin (BSA) and thyroglobulin by the glutaraldehyde technique [20]. The conjugated peptide (50 μg) was injected during the first four immunizations; for further immunizations the peptide coupled to thyroglobulin was used. Rabbits were bled 7 days after each injection. Antibody titers were determined for each bleeding for each rabbit by the ELISA system, and the highest titer antisera were selected.

To purify the antipeptide antibodies, the $\gamma_{268-282}$ peptide was coupled to CNBr-activated Sepharose 4B at the concentration of 5 mg per gram of dry resin and used for affinity chromatography. The coupling efficiency, as judged by absorbance of the peptide in the supernatant after linkage, was about 90%. The rabbit antisera with the highest antibody titers against the $\gamma_{268-282}$ were diluted 1:1 with buffer A (0.02 M sodium phosphate buffer, pH 7.5, containing 2 M NaCl, 0.02% Tween 80, 0.02 M EDTA and 0.002 M benzamidine) and adsorbed on the $\gamma_{268-282}$-Sepharose column (5 ml), then the column was washed with benzamidine-free buffer A diluted 1:1 with water. Antibodies were eluted with 0.5 M acetic acid and 2 M Tris was immediately added to each fraction to neutralize pH of the eluate. The fractions contain-

ing antibodies to $\gamma_{268-282}$ were pooled and dialysed overnight against 0.14 M NaCl buffered with 0.01 M sodium phosphate, pH 7.3. Antibodies to (FgD) of fibrinogen and their Fab fragments were characterized previously [21].

Enzyme-linked immunoabsorbent assay. Microtiter wells (Co-Bind™ activated plate) were coated with 100 µl of the $\gamma_{268-282}$ peptide at a concentration of 2 µg/ml for 2 h at 37°C. Unbound peptide was removed by rinsing with phosphate-buffered saline (PBS) and the unreacted sites were blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. The plates were then washed with PBS-0.05% Tween 20 and incubated overnight at 4°C with 100 µl aliquots of serial dilutions of antipeptide antibodies. Plates were then extensively washed with PBS-Tween 20 and incubated with a second antibody conjugated to horseradish peroxidase for 1 h. After washing, binding was quantified using *o*-phenyldiamine as a substrate and measuring the absorbance at 492 nm.

Sandwich-type enzyme immunoassay. Wells of polystyrene microtitration plates were coated with 100 µl of immunochemically purified anti-$\gamma_{268-282}$ antibodies (10 µg/ml) by overnight incubation at 4°C. The anti-$\gamma_{268-282}$ coated wells were incubated with serial dilutions of intact fibrinogen and fibrinogen treated with thrombin for different period of times. In this experiment thrombin was introduced to the final concentration of 0.05 U per mg of fibrinogen and the digestion was stopped by adding hirudin (0.5 unit) after 0, 30, and 60 s. At this stage no visible fibrin was formed, but prolonged incubation with thrombin resulted in the formation of clots as described in the next section. Captured antigen was tagged with antifibrinogen antibodies specific to the D domain (anti-FgD) which were conjugated with horseradish peroxidase [22] and visualized by incubation with *o*-phenyldiamine as a substrate.

Thrombin clotting of fibrinogen. The kinetics of thrombin-catalyzed clotting was measured by absorbance change at 350 nm as described previously [23]. Briefly, 0.5 ml aliquots of anti-$\gamma_{268-282}$, anti-FgD, their Fab fragments or $\gamma_{268-282}$ peptide in PBS were mixed with 0.5 ml of fibrinogen in the same buffer (600 µg/ml) in a quartz cell, and the base line recorded at 350 nm. The mixture was preincubated for 5 min at room temperature. Then 20 µl of thrombin (20 units/ml) was added, stirred well and the increase of absorbance was recorded for 15 min. To measure the rate of polymerization of fibrin monomers alone, 0.5 ml of fibrinogen was mixed with 0.5 ml of PBS, and 20 µl of thrombin was added. The maximum reaction rate (Δ A_{350} per minute) was calculated from the steepest portions of sigmoidal reaction profiles and expressed as percentage of maximum rate for fibrin alone.

Analytical procedures. Protein concentration was determined either by the microbiurette method [24] or by spectrophotometry at 280 nm using an absorption coefficient at 1 mg/ml, in aqueous solution at pH 7.0 of 1.5 and 1.35 for fibrinogen and IgG, respectively.

Amino acid composition was determined on a Beckman model 121 M analyzer from 24 h hydrolizates in 6.0 M HCl at 100°C *in vacuo*.

Fibrinogen, reduced and nonreduced, was separated by electrophoresis according to Laemmli [25] in slab SDS-polyacrylamide gels that were then stained with Coomassie Brillant Blue. Completed gels were also transferred electrophoretically (6 V/cm for 15 h at 4°C) onto nitrocellulose paper for staining with anti-$\gamma_{268-282}$ antibodies [26].

RESULTS

Specificity of anti-$\gamma_{268-282}$ antibodies

To determine whether the amino acids constituting the $\gamma_{268-282}$ peptide (GPEADKYRLTYAYFA) of the γ chain of fibrinogen take part in the structure of fibrin monomer polymerization site, the peptide was synthesized and purified, and anti-$\gamma_{268-282}$ antibodies were produced in rabbits and purified as described in Materials and Methods. The immuno-adsorbent assay, in which the surfaces of wells of polystyrene microtitration plates was coated with synthetic peptide corresponding to the sequence 268-282 of γ chain, showed that the latter was recognized by the anti-$\gamma_{268-282}$ (Fig. 1). Similarly, these antibodies reacted with native fibrinogen used as the antigen (not shown). On the other hand, anti-$\gamma_{268-282}$ antibodies, when used in the Western immunoblotting analysis, reacted neither with SDS-denatured fibrinogen nor its γ chain (not shown).

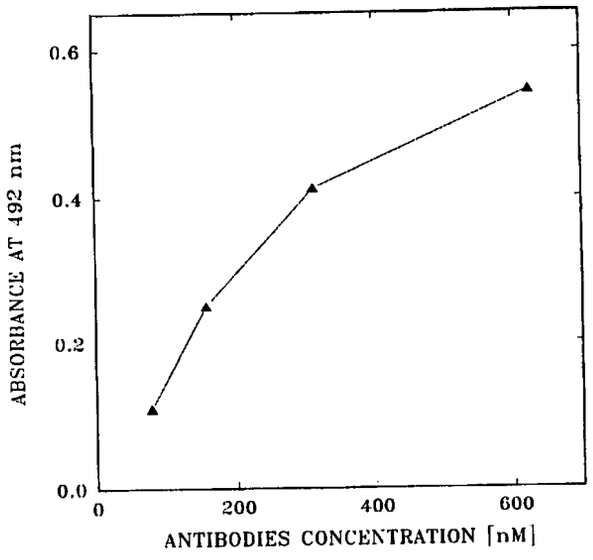

Fig. 1. Titration of anti-γ268-282 antibodies purified immunochemically by the ELISA system using microtiter plates with the synthetic peptide covalently attached to wells.

In a sandwich-type enzyme immunoassay anti-γ268-282 antibodies were able to interact with intact fibrinogen and thrombin-treated fibrinogen with the same affinity (Fig. 2). In this experiment microtitration plates coated with anti-γ268-282 antibodies were used. Virtually the same dose-response curves were obtained when fibrinogen was incubated with thrombin

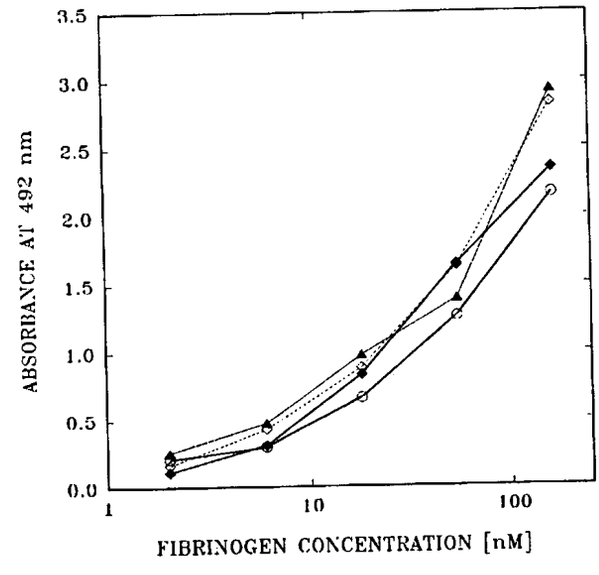

Fig. 2. The binding of intact fibrinogen (○) and thrombin-treated fibrinogen for: 10 s (■), 30 s (❑), and 60 s (▲) to anti-γ268-282 adsorbed on the surface of microtiter plates.
Fibrinogen bound was detected by applying anti-FgD antibodies conjugated with peroxidase, and quantitated.

for 0, 30 or 60 s indicating that the recognized epitope was exposed on the intact fibrinogen and its expression was not influenced by thrombin digestion.

The effect of γ268-282 peptide and antipeptide antibodies on fibrin monomer polymerization

The inhibition of thrombin-catalyzed conversion of fibrinogen to fibrin produced by anti-FgD antibodies, their Fab fragments and anti-γ268-282 antibodies is compared in Fig. 3. In contrast to anti-FgD antibodies and their fragments which showed strong antipolymerizing activity, anti-γ268-282 antibodies had no effect on thrombin-induced fibrin monomer polymerization. The same negative effect was observed when thrombin-catalysed clotting of fibrinogen was carried out in the presence of 500-fold molar excess of the γ268-282 peptide (not shown).

DISCUSSION

Molecular defects of fibrinogen which affect release of the fibrinopeptides A or B or fibrin

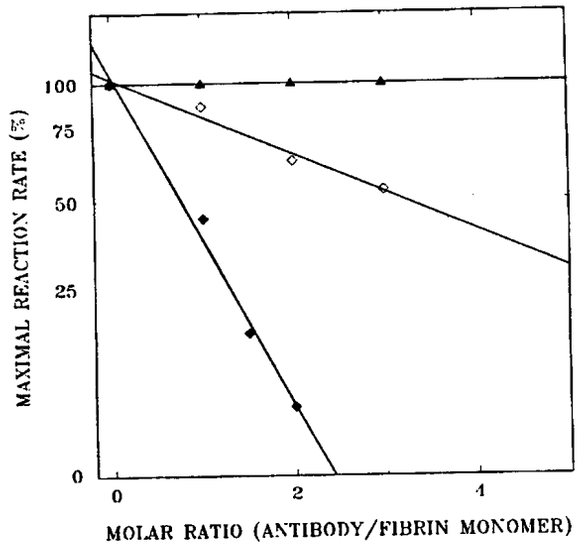

Fig. 3. The effect of anti-γ268-282 (▲) and anti-FgD (■) antibodies on fibrin monomer polymerization. In addition, the inhibitory effect of Fab fragments obtained from anti-FgD antibodies is shown (❑).
Fibrinogen was mixed with increasing concentrations of antibodies or Fab fragments and then thrombin was added. Formation of fibrin was monitored spectrophotometrically at 350 nm. The maximum reaction rate (ΔA_{350}/min) was calculated from the steepest portion of sigmoidal reaction profiles and expressed as percentage of the rate for fibrin alone.

monomer polymerization might be manifested clinically as prolongation of clotting times, such as the prothrombin, activated partial thromboplastin, or thrombin times [27]. A number of congenitally abnormal fibrinogens with impaired fibrin assembly were found to have single amino acid substitutions localized in the γ chain positions Arg^{275} [5, 10 - 14], Gly^{292} [28], Asn^{308} [29, 30], Met^{310} [31], Gln^{329} [13] and Asp^{330} [32]. Since these natural mutations produced fibrinogen molecules with impaired ability to polymerize, the polymerization site originally postulated to be present within the $\gamma_{357-411}$ segment [6], recently was proposed to be located in sequences starting from Arg^{275} [7, 9, 29]. Identification of specific amino-acid residues involved in the structure of the polymerization site is technically difficult to perform. Although a large segment of γ chain corresponding to the sequence 259-411 was expressed in *E. coli* and obtained as a recombinant peptide fragment, it cannot be used in functional studies due to its poor solubility [33].

Application of antipeptide antibodies in studies of the structure-function relationship in fibrinogen molecule can be very instructive [34, 35]. Anti-$\gamma_{268-282}$ used in this study was found to react with the epitope present in the synthetic peptide $\gamma_{268-282}$, in the native fibrinogen molecule and fibrin monomers, indicating that this epitope is exposed on the hydrated surface of fibrinogen molecule. However, attachment of the antibody molecule to this region did not inhibit fibrin monomer polymerization, proving that Arg^{275} and contiguous sequences are neither directly nor indirectly involved in the structure of the polymerization site. Thus, the impaired fibrin assembly caused by substitution of the Arg^{275} residue by His in abnormal fibrinogens Haifa I [10], Bergamo II [11], Essen I [11], Perugia I [11], and Saga I [12] or by Cys in abnormal fibrinogens Tochigi I [13], Osaka II [5], and Baltimore IV [14] must have resulted from a mechanism other than direct modification of polymerization site.

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