Fibrinogen Biosynthesis in Isolated Guinea Pig Megakaryocytes

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Fibrinogen synthesis was investigated in guinea pig megakaryocytes. Purified megakaryocytes were incubated with $^{35}$S-methionine in methionine-free incubation medium for 18 hours. Newly synthesized fibrinogen in megakaryocyte lysates enriched with purified carrier guinea pig fibrinogen was immunoprecipitated with a specific anti-guinea pig fibrinogen antisera produced in rabbits. Proteins in the immunoprecipitates were analyzed with a 3.5% to 10.0% gradient polyacrylamide slab gel electrophoresis and autoradiography. Reactivity was detected in a protein band of 340,000 daltons. To verify fibrinogen synthesis, immunoprecipitate was analyzed by two-dimensional slab gel electrophoresis: (1) the first dimension separated unreduced fibrinogen using a 3.5% to 10.0% gradient gel; (2) following reduction by 2-$\beta$-mercaptoethanol, fibrinogen chains were separated in the second dimension using a 10% gel. Alpha, beta, and gamma fibrinogen chains, which represented carrier guinea pig plasma fibrinogen, were visualized by Coomassie brilliant blue. Autoradiography identified the incorporation of radioactivity into the three fibrinogen chains. In control experiments, immunoprecipitates, produced by exposing megakaryocyte lysates to preimmune rabbit serum and goat anti-rabbit IgG, were also analyzed by the two-dimensional gel system. Radioactivity was not detected in sites corresponding to the migration of fibrinogen subunits. The study demonstrates that isolated guinea pig megakaryocytes can synthesize fibrinogen. The electrophoretic mobility of newly synthesized fibrinogen and subunits is similar to that of guinea pig plasma fibrinogen.

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Materials and Methods

Megakaryocytes were isolated from guinea pig bone marrow of up to 80% purity by cell number and 97% purity by cell volume by the method of Levine and Fedorko and others. About 10$^7$ purified megakaryocytes were incubated with 200 $\mu$Ci of $^{35}$S-methionine ($\sim$600 Ci/mmol), 5.123. Amersham (Arlington Heights, Ill) using Dulbecco’s modified Eagle’s medium with glutamine, penicillin, and streptomycin (Gibco, Grand Island, NY). The cells were incubated for 18 hours in 5% CO$_2$-95% air at 37°C in a CO$_2$ incubator. After the incubations, the megakaryocytes were washed three times with phosphate-buffered saline (PBS), pH 7.4, with EDTA (1.0 mmol/L), phenylmethylsulfonyl fluoride (1.0 mmol/L), and aprotinin (100 kallikrein inhibitor units [KIU]/mL). Washed cells were resuspended in PBS, pH 7.4, with 0.1% Triton X-100 and were disrupted by freezing and thawing three times. Insoluble material was separated from the cell lysate by centrifugation at 15,600 g for five minutes.

In order to immunoprecipitate guinea pig fibrinogen in the cell lysates, purified guinea pig fibrinogen (0.1 mg/mL) and anti-guinea pig fibrinogen antisera (1:10 dilution) were added to the cell lysates. The immunoprecipitates were pelleted by centrifugation at 1,200 g for five minutes. The addition of preimmune rabbit serum and goat anti-rabbit IgG to the cell lysates served as preimmune controls. The immunoprecipitates were exhaustively washed with the buffer used for cell washes and then solubilized in 2% sodium dodecyl sulfate (SDS) Tris buffer, pH 6.8, with 10% sucrose.

In order to isolate fibrinogen, immunoprecipitates were solubilized and separated by slab gel electrophoresis (LKB, Gaithersburg, Md). One-dimension gels, performed by a modification of the method of Laemmli, were used to separate unreduced proteins. Two-dimension gels were carried out under reduced conditions to resolve the individual polypeptide chains. The gels were stained with Coomassie brilliant blue and newly synthesized fibrinogen was detected by subjecting the gels to autoradiography using Kodak (Rochester, NY) XAR-5 film.

Fibrinogen was purified from guinea pig plasma by the following methods: Guinea pig citrated plasma was diluted with an equal volume of water and brought to 21% saturation (1.59 mol/L) with solid ammonium sulfate. The precipitate that formed was extracted twice for one hour in 0.85 mol/L ammonium sulfate and 0.025 mol/L sodium citrate. The precipitate was dissolved in 0.15 mol/L sodium chloride, 0.025 mol/L sodium citrate, and 0.01 mol/L sodium citrate, and then dialyzed overnight.

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sodium phosphate buffer, pH 7.0. The solution was cooled on ice, then clarified by centrifugation. Solid ammonia-free glycine was added to a final concentration of 2.1 mol/L. The fibrinogen precipitate was pelleted and dissolved in 0.15 mol/L sodium chloride, 0.025 mol/L sodium citrate, 0.05 mol/L Tris-hydroxymino methane, pH 7.4, and kept in aliquots at -20°C. Unreduced and reduced fibrinogen samples were analyzed by disc gel polyacrylamide electrophoresis with 3.5% and 7.0% gels, respectively. 2-mercaptoethanol (1%) was used for the reduction of fibrinogen. Polyclonal anti-guinea pig antibodies were raised by the immunization of New Zealand white rabbits (Hazelton Research Labs, Denver, Pa). The antiserum was absorbed by successive additions of 10 mg of lyophilized guinea pig serum per milliliter of antiserum. The specificity of the absorbed anti-guinea pig fibrinogen antibodies was determined by immunoelectrophoresis.

RESULTS

Purified guinea pig fibrinogen was characterized by using disc gel electrophoresis, which demonstrated that the molecular weights of unreduced and reduced fibrinogen chains were similar to that of human fibrinogen. The yield of fibrinogen was 0.5 to 0.7 mg/mL of guinea pig plasma.

The immunoelectrophoretic pattern shown in Fig 1 demonstrates the presence of a single precipitin arc between anti-guinea pig fibrinogen antiserum and either purified guinea pig fibrinogen or plasma. The monospecificity of the antibody is demonstrated by the absence of a precipitin arc between preimmune serum and guinea pig plasma.

Incorporation of 35S-methionine into fibrinogen was demonstrated in five experiments. A representative experiment in which immunoprecipitated fibrinogen was analyzed by one-dimension polyacrylamide gel electrophoresis is shown in Fig 2. Two protein bands are seen in the Coomassie brilliant blue-stained gel, a single band of about 160,000 daltons, which represents IgG, and another band of about 340,000 daltons corresponding to that of fibrinogen. The autoradiogram (35S) of the gel indicated that radioactivity was present in the fibrinogen band and represented newly synthesized protein.

In order to definitively identify fibrinogen synthesis, we demonstrated the incorporation of 35S-methionine into the three fibrinogen chains in four experiments. This was achieved by performing two-dimension slab gel electrophoresis. A representative experiment is shown in Fig 3. The gel shown in Fig 3A had been stained with Coomassie brilliant blue. The first dimension was carried out under nonreducing conditions and shows the separation of fibrinogen from IgG. In the second dimension, reduced fibrinogen chains are separated from the IgG heavy chain. Figure 3B represents an autoradiogram of the same gel and shows that 35S-methionine had been incorporated into the α-, β-, and γ-chains of fibrinogen. It was also apparent that the newly synthesized fibrinogen and fibrinogen chains had comigrated with, and had the same molecular weight as, guinea pig plasma fibrinogen that had been used as an internal standard.
FIBRINOGEN SYNTHESIS IN MEGAKARYOCYTES

Fig 3. Electrophoretic characteristics and synthesis of fibrinogen chains: Two-dimension acrylamide slab gel electrophoresis. Following the incubation of isolated megakaryocytes with [35S]-methionine for 18 hours, megakaryocytes were lysed and carrier-purified guinea pig plasma fibrinogen was added. Next, fibrinogen in the lysates was immunoprecipitated with anti-guinea pig fibrinogen antiserum and analyzed by two-dimension slab gel electrophoresis, unreduced fibrinogen was separated by using a 3.5% to 10.0% gradient gel in the first dimension, and following reduction by 2-mercaptoethanol (1%) for 30 minutes, fibrinogen chains were separated by a 10% gel. (A) shows a Coomassie brilliant blue-stained gel and (B) demonstrates the autoradiogram of the gel. (C) and (D) demonstrate the control experiment for that shown in (A) and (B). Preimmune immunoprecipitation was achieved by the addition of preimmune rabbit serum and goat anti-rabbit IgG to the megakaryocyte lysates and was analyzed by the same gel electrophoretic system as used in (A) and (B). IgG HC represents the immunoglobulin heavy chain, IgG LC represents the light chains, and α, β, and γ represent alpha, beta, and gamma subunits of fibrinogen.

It is apparent that other proteins have been trapped in the immunoprecipitate as shown in Fig 3A and that these proteins have incorporated radioactivity as shown in Fig 3B. Figure 3C represents a Coomassie brilliant blue-stained two-dimension gel of a preimmune immunoprecipitate and Fig 3D shows a corresponding autoradiogram. The background pattern of trapped proteins and evidence of incorporated radiolabeled methionine, shown in Fig 3C and D, resembled that shown in Fig 3A and B. However, there was no radioactivity present in the area of the gel in which fibrinogen chains would migrate, as shown in the autoradiogram shown in Fig 3D.

We attempted to decrease the nonspecific trapping of proteins described above by several approaches, ie, precipitation of nonspecifically labeled proteins with preimmune serum and goat anti-rabbit IgG prior to the immunoprecipitation of fibrinogen. These measures were effective but significantly reduced the recovery of radiolabeled fibrinogen. This problem may account for the difficulty in the demonstration of fibrinogen synthesis in megakaryocytes.

This is the first study that demonstrates fibrinogen biosynthesis in isolated guinea pig megakaryocytes. Preliminary report has suggested that human megakaryocytes can synthesize fibrinogen.16 Fibrinogen is known to be present in substantial amounts in megakaryocytes9 and most likely is stored in megakaryocyte organelles that resemble α-granules. Therefore, fibrinogen, like factor VIII:Ag,7 factor V,5 and platelet factor 4,10 can be synthesized by megakaryocytes and stored in platelet α-granules.

The evidence that platelet fibrinogen may originate in the megakaryocyte does not exclude the possibility that platelet and plasma fibrinogen are identical gene products. Our study suggests that megakaryocyte and plasma fibrinogen are identical gene products, based on the demonstration that the electrophoretic mobility of the newly synthesized fibrinogen chains and plasma fibrinogen were identical. However, the analytical gel techniques used in our study cannot show small structural differences, such as posttranslational glycosylation of Bβ- and γ-chains, which could alter the properties of megakaryocyte fibrinogen.

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Fibrinogen biosynthesis in isolated guinea pig megakaryocytes

RM Leven, PK Schick and AZ Budzynski