and ATP was readily calculated if the ligands were in large excess, and concentration ratios r_{Phe} and r_{ATP} were computable from eq 3 and 6, respectively:

$$r_{Phe} = \frac{[Phe^{-}Zn^{2+}]}{[Zn^{2+}]} \qquad r_{ATP} = \frac{[ATP{\cdot}Zn^{2+}]}{[Zn^{2+}]} \tag{7}$$

Insertion into the approximate conservation equation

$$[Zn^{2+}]_0 - [Zn^{2+}] = [Phe^{-}Zn^{2+}] + [ATP{\cdot}Zn^{2+}] \tag{8}$$

gives

$$[Zn^{2+}] = [Zn^{2+}]_0 \frac{1}{1 + r_{Phe} + r_{ATP}} \tag{9}$$

Registry No. Ap_4A, 5542-28-9; TNS, 7724-15-4; Phe, 63-91-2; MgATP, 1476-84-2; Zn, 7440-66-6; phenylalanyl-tRNA synthetase, 9055-66-7; phenylalanyladenylate, 35874-27-2; lysyl-tRNA synthetase, 9031-26-9; pyruvate kinase, 9001-59-6; arginyl-tRNA synthetase, 37205-35-9; isoleucyl-tRNA synthetase, 9030-96-0; tryptophanyl-tRNA synthetase, 9023-44-3; tyrosyl-tRNA synthetase, 9023-45-4; aldolase, 9024-52-6; alcohol dehydrogenase, 9031-72-5; catalase, 9001-05-2; tryptophan synthase, 9014-52-2.

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In Vitro Biosynthesis of Plasminogen in a Cell-Free System Directed by mRNA Fractions Isolated from Monkey Liver†

Mario Gonzalez-Gronow* and Kenneth C. Robbins

ABSTRACT: mRNA was isolated from total RNA of monkey liver by oligo(dT)–cellulose chromatography and was translated in a rabbit reticulocyte cell-free system. Analysis of the translation products immunoprecipitated with specific antibodies to monkey plasma plasminogen revealed a molecule with characteristics similar to those of native plasminogen. The purification of the mRNA by centrifugation on sucrose gradients indicated the presence of plasminogen mRNAs in both the 23S and 18S RNA fractions. Both plasminogen mRNAs can be further purified by chromatography on Sepharose 4B. Affinity chromatography of the proteins synthesized in vitro by total mRNA from liver, as well as by the purified mRNAs, on L-lysine-substituted Sepharose revealed that both major plasma plasminogen forms (1 and 2) are synthesized, as precursors, in the system. The in vitro synthesized plasminogen is similar in its physical and chemical properties to native plasma plasminogen as determined by its ability to bind to L-lysine-substituted Sepharose and its molecular interaction with streptokinase. The purified mRNAs were also translated in the presence of dog pancreas microsomal membranes, and the proteins sequestered inside the membranes were released and fractionated on concanavalin A–Sepharose. The 23S mRNA directed the synthesis of a plasminogen molecule similar to the circulating plasma plasminogen form 1, whereas the 18S mRNA directed the synthesis of a molecule similar to the circulating plasma plasminogen form 2. Our evidence indicates that the synthesis of the two major circulating plasma plasminogen forms is directed in the liver by separate mRNAs.

Plasminogen, the plasma zymogen of the fibrinolytic enzyme plasmin, is a single-chain protein with a molecular weight of about 88 000 containing 2% carbohydrate. It consists of 790 amino acids of known sequence (Sottrup-Jensen et al., 1978). Plasminogen is isolated from plasma by affinity chromatography on L-lysine-substituted Sepharose (Deutsch & Mertz, 1970) as two major forms (Brockway & Castellino, 1972) which differ in their states of glycosylation (Hayes & Castellino, 1979a–c). The active enzyme plasmin consists of two chains held together by disulfide bonds which are formed when the Arg_{560}–Val peptide bond of plasminogen is cleaved by a

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class of highly specific proteases known as plaminogen activators (Robbins et al., 1967).

While the chemistry of the plasminogen molecule and the processes of its activations have been characterized in great detail, very little is known about its metabolism. Recent studies (Knowles et al., 1980) demonstrated that the cell culture fluid from two human hepatoma-derived cell lines contains plasminogen along with 17 of the major human plasma proteins synthesized and secreted by these cells. Also, studies performed with primary monolayer cultures of rat parenchymal hepatocytes have clearly demonstrated that both plasminogen forms are synthesized and secreted at rates sufficient to account for the majority of the in vivo plasminogen turnover (Bohmfalk & Fuller, 1980). These observations have been confirmed in studies on isolated perfused rat liver (Saito et al., 1980), in which the rate of release of plasminogen indicates that the liver is the predominant site of in vivo plasminogen production.

The liver is an excellent tissue for the study of the synthesis and secretion of plasma proteins. Several mRNAs have been isolated from liver and used to direct the in vitro synthesis of albumin, fibrinogen, prothrombin, and antithrombin III (Taylor & Tse, 1976; Gordon et al., 1978; MacGillivray et al., 1979). In the present study, we describe the isolation of two mRNA populations from monkey liver, which after translation in a cell-free protein-synthesizing system directed the synthesis of molecules which once segregated inside dog pancreas microsomal membranes were identified as the two major circulating plasma plasminogen forms.

Materials and Methods

Proteins. Plasminogen was purified from fresh monkey and human plasma by an affinity chromatography method with L-lysine-substituted Sepharose (Deutsch & Mertz, 1970).

Plasminogen Antibodies. One milligram of purified human Glu-plasminogen was dissolved in 0.5 mL of 0.005 M sodium phosphate/0.15 M sodium chloride buffer, pH 7.4, and was emulsified with 1 mL of Freund's complete adjuvant. The emulsion was injected subcutaneously into goats and was followed by booster doses in Freund's incomplete adjuvant at weekly intervals. Two weeks following the third injection, the animals were bled, and the antiserum was collected. The specific antibodies to monkey plasminogen were isolated from this antiserum by passing the serum through a monkey plasminogen–Sepharose affinity column followed by elution of the antibodies with 0.5 M acetic acid. Plasminogen was coupled to CNBr-activated Sepharose at pH 8.5 by the method of Cuatrecasas (1970). The antibodies prepared by this procedure were not inhibitory to protein synthesis in a reticulocyte lysate translation system.

Radioiodination of Plasminogen. Radioiodination was carried out by the method of Hunter (1978). One milligram of monkey plasminogen was dissolved in 0.5 mL of 0.05 M tris(hydroxymethyl)aminomethane (Tris)/0.15 M sodium chloride buffer, pH 7.4, in a 13 × 100 mm test tube, and the solution was stirred in an ice bath for several minutes. $Na^{125}I$ (3 mCi) was added and was followed by the addition of 0.03 mL of a solution containing 5 mg/mL Chloramine-T in 0.05 M sodium phosphate buffer, pH 7.4. Approximately 1 min later, 0.05 mL of sodium metabisulfite (5 mg/mL) in the pH 7.4 sodium phosphate buffer was added. The contents of the tube were passed through a Sephadex G-25 column (1.5 × 20 cm) and washed with the pH 7.4 Tris buffer; 1-mL fractions were collected. The labeled protein was located by taking 5-μL aliquots from each tube and adding each to 2 mL of Insta-Gel (Packard). The samples were counted in a Packard Tri-Carb 460 CD liquid scintillation counter using a program for ^{125}I. The labeled protein was stored frozen at −20 °C. Incorporation of ^{125}I was approximately 8×10^6 cpm/nmol of plasminogen.

Preparation of mRNA. Livers from monkeys were obtained within 5 min after death, and excess blood was removed by rinsing the livers in sterile phosphate-buffered saline (0.01 M sodium phosphate/0.15 M sodium chloride, pH 7.2). The liver was then cut into 0.5-cm slices, rinsed in sterile phosphate-buffered saline, and frozen in liquid nitrogen. The method of Kirby (1968) was used to isolate total RNA. Twenty grams of frozen tissue was broken down in a blender with 400 mL of a mixture containing 200 mL of 6% 4-aminosalicylate/1% sodium chloride solution, 150 mL of liquefied phenol, and 50 mL of *m*-cresol. The mixture was stirred for 20 min at 20 °C and then centrifuged at 6000*g* for 30 min at 5 °C. Sodium chloride (3 g) was added to each 100 mL of aqueous phase, which was then reextracted 2 times with the phenol/cresol mixture. Total RNAs were then precipitated with 2 volumes of cold 95% ethanol. The precipitate was centrifuged off and extracted 2 times with cold 3 M sodium acetate buffer, pH 6.0 (30 mL each time), washed once with a cold mixture of water (25 mL), sodium chloride (1 g), and 95% ethanol (75 mL) and twice with 95% ethanol, and then dried in a vacuum desiccator over $CaCl_2$. The yield was about 70 mg of total RNA. The poly(A)-containing mRNA was isolated by binding the total RNA to an oligo(dT)–cellulose column followed by elution with distilled water (Aviv & Leder, 1972). The mRNA (2 mg) was then precipitated with 2 volumes of cold 95% ethanol and redissolved in sterile distilled water.

Sucrose Gradient Centrifugation. The mRNA was fractionated according to molecular size by centrifugation on linear sucrose density gradients. The gradient of 5–20% (w/v) sucrose was made in a 50% solution of formamide and buffer containing 0.1 M Tris/0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.5. Before application to the gradients, the mRNA samples (2–5 A_{260} units) were dissolved in 200 μL of 50% formamide, incubated for 2 min at 37 °C, and quickly cooled on ice. After centrifugation (rotor SW 55Ti, Beckman) at 36 000 rpm, for 18 h at 15 °C, the gradients were tapped from the bottom in 250-μL fractions. The absorbance at 260 nm was measured and the mRNA in each fraction precipitated by the addition of cold 95% ethanol and stored at −20 °C.

Sepharose 4B Chromatography. The mRNA was chromatographed on a Sepharose 4B (1.5 × 15 cm) column equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.65 M sodium chloride/0.002 M EDTA (Frischauf et al., 1978). The bound mRNA was eluted from the column by using 0.02 M Tris-HCl buffer, pH 7.5, containing 0.1 M sodium chloride/0.002 M EDTA. Both the unbound and bound mRNA fractions were precipitated with cold 95% ethanol.

Cell-Free Translation of mRNA. Translation of mRNA in vitro was carried out by employing an mRNA-dependent reticulocyte lysate system as described by Pelham & Jackson (1976). The concentration of K^+ and Mg^{2+} in the system was adjusted when necessary to compensate for the dilution by the other components of the reaction mixture. A typical translation was carried out for 1 h at 30 °C; the system contained 10 μL of mRNA (5 μg), 50 μL of reticulocyte lysate, and 40 μL of translation cocktail containing 30–50 μCi of [^{35}S]-methionine. The ability of the mRNA to direct protein synthesis is determined by the mRNA-dependent incorporation of [^{35}S]methionine into hot (100 °C) trichloroacetic acid insoluble material.

Microsomal Membranes. A dog pancreas (20 g) was used

for the preparation of microsomal membranes according to the procedure of Shields & Blobel (1978). The tissue was homogenized with 100 mL of 0.01 M Tris/0.15 M sodium chloride/0.25 M sucrose buffer, pH 7.4, and a postmitochondrial supernatant (PMS) prepared by centrifuging the homogenate at 8000*g* for 10 min. The microsomal membranes were then pelleted by centrifuging the PMS at 100000*g* for 1 h. The pellet was then resuspended in homogenization buffer, layered over cushions of 1.3 M sucrose containing 0.005 M magnesium chloride/0.05 M potassium chloride/0.05 M Tris buffer, pH 7.4, and centrifuged for 90 min at 100000*g*. The pellet was then stripped of ribosomes by resuspending it in 0.25 M sucrose/0.1 M potassium chloride/0.02 M Tris buffer, pH 7.4, containing 0.002 M EDTA. After 20 min at 4 °C, the suspension was layered over cushions of 20% sucrose containing 0.1 M potassium chloride/0.02 M Tris buffer, pH 7.4, and centrifuged for 1 h at 110000*g*. This pellet constituting stripped microsomes was stored at −80 °C until further use.

In Vitro Segregation and Glycosylation of Plasminogen. The purified plasminogen mRNA fractions were translated with a rabbit reticulocyte lysate in the presence of stripped microsomes prepared as described above. We used 20 A_{260} units of microsomes per mL of translation mixture. The system was optimized for glycosylation by adding 250 μM GDP–mannose/3.5 mM UDP–*N*-acetylglucosamine/250 μM UDP–galactose/100 μM CMP–*N*-acetylneuraminic acid, as described by Czichi & Lennarz (1977). After incubation for 90 min at 30 °C, the translation mixture was layered over cushions of 20% sucrose containing 0.1 M potassium chloride/0.02 M Tris buffer, pH 7.4, and centrifuged for 1 h at 110000*g*. The pelleted membranes were then resuspended in 0.2% Triton X-100 in phosphate-buffered saline to release the proteins sequestered inside. This solution was then adsorbed to concanavalin A–Sepharose, and the bound proteins were eluted with 0.2 M methyl α-mannoside in phosphate-buffered saline. Both the bound and unbound protein fractions were then subjected to immunoprecipitation with purified anti-monkey plasminogen antibodies.

Immunoprecipitation of the in Vitro Synthesized Plasminogen. Specific immunoprecipitation of plasminogen was carried out by using purified antibodies to monkey plasminogen. Five micrograms of goat anti-monkey plasminogen antibodies was added to 50 μL of cell-free translation mixture, or 100 μL of resuspended and lysed microsomal membranes after segregation as described above. After 2 h at room temperature, 10 μg of rabbit antibody to goat immunoglobulin G (IgG) was added, and the mixture was kept overnight at 4 °C. The immunoprecipitate was collected by centrifugation and washed 2 times with phosphate-buffered saline, pH 7.4, and once with distilled water; it was dissolved in 40 μL of 4 M urea/1% sodium dodecyl sulfate (SDS)/1% mercaptoethanol/0.1 M Tris buffer, pH 8.8, and heated at 50 °C for 15 min. The sample was applied directly to the polyacrylamide slab gel.

Electrophoretic Analysis. SDS–polyacrylamide gel electrophoretic analysis was carried out according to Laemmli (1970) on an 8% polyacrylamide slab gel in 0.1% SDS/0.1 M Tris–glycine buffer, pH 8.6. After electrophoresis at 100 V for 4 h, the gels were equilibrated with dimethyl sulfoxide, impregnated with 2,5-diphenyloxazole (PPO) by immersion in 20% PPO in dimethyl sulfoxide (w/w), soaked in water, dried, and exposed to Kodak X-Omat film at −70 °C (Laskey & Mills, 1975). Exposed films were developed for 5 min at 20 °C in Kodak DX-80.

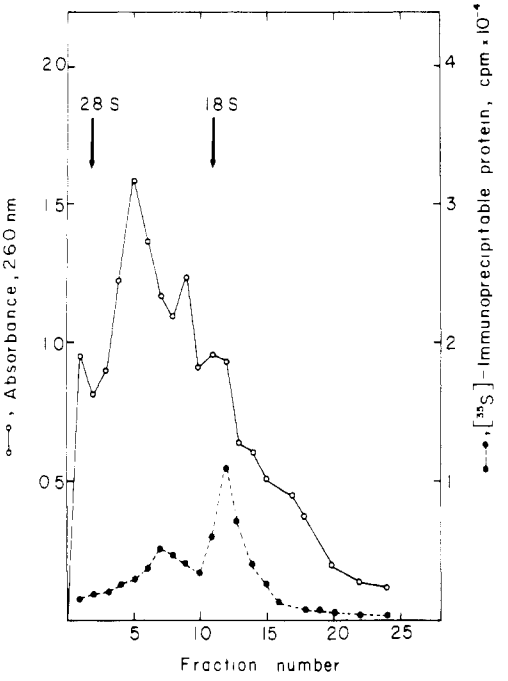

FIGURE 1: Separation by sucrose gradient centrifugation of oligo(dT)–cellulose-purified mRNA from monkey liver. The RNA (5 A_{260} units) was loaded as described under Materials and Methods and the sedimentation profile measured at 260 nm (○). Alliquots (0.5 μg of RNA) of every fraction were translated in the presence of [^{35}S]-methionine and the products of translation immunoprecipitated with monkey plasminogen antibodies (●).

Results

The mRNA from monkey liver isolated by oligo(dT)–cellulose chromatography was fractionated by centrifugation in a preparative sucrose gradient as described under Materials and Methods. Equal amounts (0.5 μg) of every fraction were translated in a rabbit reticulocyte system using [^{35}S]methionine as a label, and the proteins synthesized in vitro were immunoprecipitated with specific monkey plasminogen antibodies. Figure 1 shows the sedimentation profile and the resulting plasminogen activity of these mRNA fractions. We found plasminogen in the region of 18S and 23S mRNAs. The fractions with plasminogen mRNA activity were pooled and the concentrations adjusted to 1 μg/mL. After translation, the polyacrylamide gel electrophoretic analysis of the immunoprecipitated protein revealed that both mRNA fractions direct the synthesis of a protein with electrophoretic mobility similar to that of monkey plasminogen as shown in Figure 2.

The mRNA fractions obtained after sedimentation on sucrose gradients were also fractionated on Sepharose 4B as described by Frischauf et al. (1978). The chromatography of total liver mRNA presented plasminogen activity on both the bound and unbound fractions, as shown after immunoprecipitation of their translated products in Figure 3a. The 23S mRNA pool obtained after sedimentation on a sucrose gradient revealed plasminogen mRNA activity only on the bound fractions, as seen in Figure 3b, whereas the 18S mRNA pool shows plasminogen activity only on the unbound fractions, as seen in Figure 3c. The electrophoretic analysis of the proteins immunoprecipitated after translation on both peaks revealed the presence of polypeptides similar to the ones shown in Figure 2 for the 23S and 18S mRNAs.

In order to determine whether the plasminogen synthesized in vitro had physicochemical properties similar to those of native plasma plasminogen, we utilized a unique property of plasminogen, namely, binding to L-lysine-substituted Sepharose and elution by 6-aminohexanoic acid. As seen in Figure 4b,

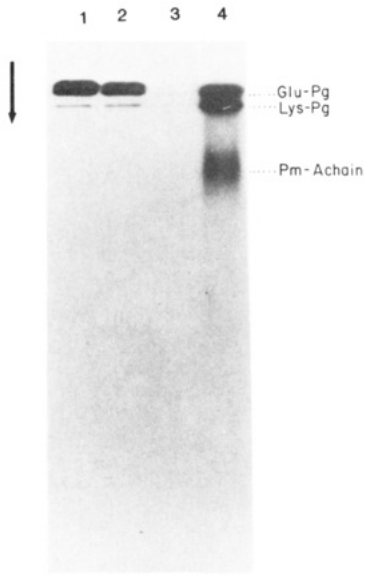

FIGURE 2: SDS–polyacrylamide gel analysis of in vitro synthesized plasminogen. 10-μg mRNA fractions separated by sucrose gradient centrifugation were translated in a 100-μL reaction mixture containing [^{35}S]methionine as the labeled amino acid. After translation, the plasminogen was immunoprecipitated as described under Materials and Methods. The immunoprecipitate was dissolved in 40 μL of 4 M urea/1% SDS/1% mercaptoethanol/0.1 M Tris buffer, pH 8.8, and applied on top of a SDS–polyacrylamide gel (8%) followed by fluorography. Lane 1, immunoprecipitate after translation with 23S mRNA; lane 2, immunoprecipitate after translation with 18S mRNA; lane 3, immunoprecipitate after translation with no liver mRNA added; lane 4, monkey ^{125}I-Glu-plasminogen, ^{125}I-Lys-plasminogen, and ^{125}I-plasmin.

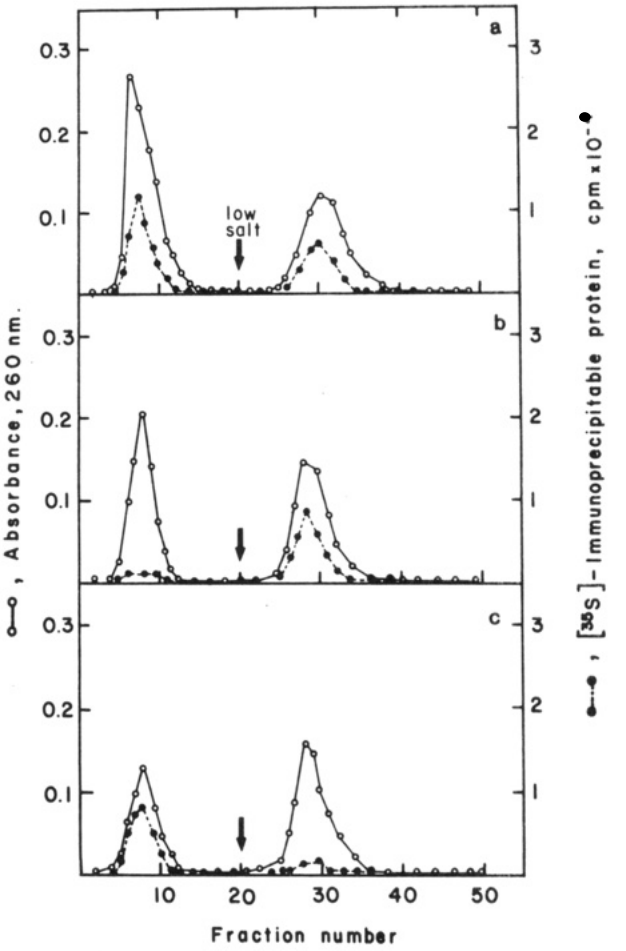

FIGURE 3: Sepharose 4B chromatography of the mRNA fractions separated by sedimentation on sucrose gradients. Absorbance profile at 260 nm (O) of total mRNA after oligo(dT)–cellulose (a); pool 23S mRNA from sucrose gradient (b); pool 18S mRNA from sucrose gradient (c). Of each fraction, 1 μg was translated in the presence of [^{35}S]methionine, and their products were immunoprecipitated with antibodies to monkey plasminogen (●) as described under Materials and Methods.

when the reaction mixture after translation with total liver mRNA in the presence of [^{35}S]methionine was filtered through a column of L-lysine-substituted Sepharose, the material adsorbed to the column, and when eluted with a gradient of 6-aminohexanoic acid, gave two radioactive peaks similar to the two major plasma forms (1 and 2) obtained with native monkey plasminogen purified under similar conditions, as seen in Figure 4a. The material adsorbed after synthesis with the mRNA bound to the Sepharose 4B (23S mRNA) revealed a peak which eluted in the same position as the plasminogen form 1, as shown in Figure 4c. The material adsorbed after synthesis with the mRNA unbound to Sepharose 4B (18S mRNA) revealed a peak which eluted in the same position as the plasminogen form 2, as shown in Figure 4d.

Since the newly synthesized plasminogen behaved similarly to native plasma plasminogen with regard to its affinity for L-lysine-substituted Sepharose, we studied the ability of this molecule to bind to ^{125}I-streptokinase. The cell-free synthesizing system was incubated with nonlabeled amino acids. After translation, the reaction mixture was incubated with ^{125}I-streptokinase and filtered through the L-lysine-substituted Sepharose column. As seen in Figure 5, the only material eluted with 0.2 M 6-aminohexanoic acid was a single peak of ^{125}I radioactivity, indicating the presence of a complex between the newly synthesized plasminogen and streptokinase.

It has been demonstrated that after chromatography on concanavalin A–Sepharose only native human plasminogen form 1 binds to the lectin, while human plasminogen form 2 does not bind to this resin. This differential binding has been assumed to be due to the presence of a higher content of mannose in plasminogen form 1 than in plasminogen form 2 (Kottgen et al., 1982). We adsorbed separately native monkey plasminogen forms purified on L-lysine-substituted Sepharose to concanavalin A–Sepharose and found that only plasminogen form 1 binds to this resin, as seen in Figure 6a. Monkey plasminogen form 2 does not bind to concanavalin A–Sepharose, as shown in Figure 6b. We translated separately the mRNAs for the two major plasminogen forms in the presence of dog pancreas microsomal membranes optimized for glycosylation. After translation, the membranes were separated from the translation mixture, and the glycosylated proteins sequestered inside were released by treatment with 0.2% Triton X-100 in phosphate-buffered saline and then filtered through a concanavalin A–Sepharose column. Panels c and d, respectively, of Figure 6 show the results of the chromatography on concanavalin A–Sepharose of the segregated and glycosylated proteins synthesized with the 23S and 18S mRNAs. The 23S mRNA directs the synthesis of the plasminogen form 1, as seen in Figure 6c, after immunoprecipitation of the proteins bound to concanavalin A–Sepharose with specific monkey plasminogen antibodies. The 18S mRNA directs the synthesis of plasminogen form 2, as seen in Figure 6d, after immunoprecipitation of the fractions not bound to concanavalin A–Sepharose. Figure 7 shows that both mRNA fractions direct the synthesis of plasminogen molecules with electrophoretic mobilities similar to that of native plasminogen. Lanes 1 and 2 are the immunoprecipitates of plasminogen synthesized in the absence of microsomal membranes, indicating the presence of precursor forms with molecular weights slightly higher than native plasminogen. Lanes 3 and 4 are the im-

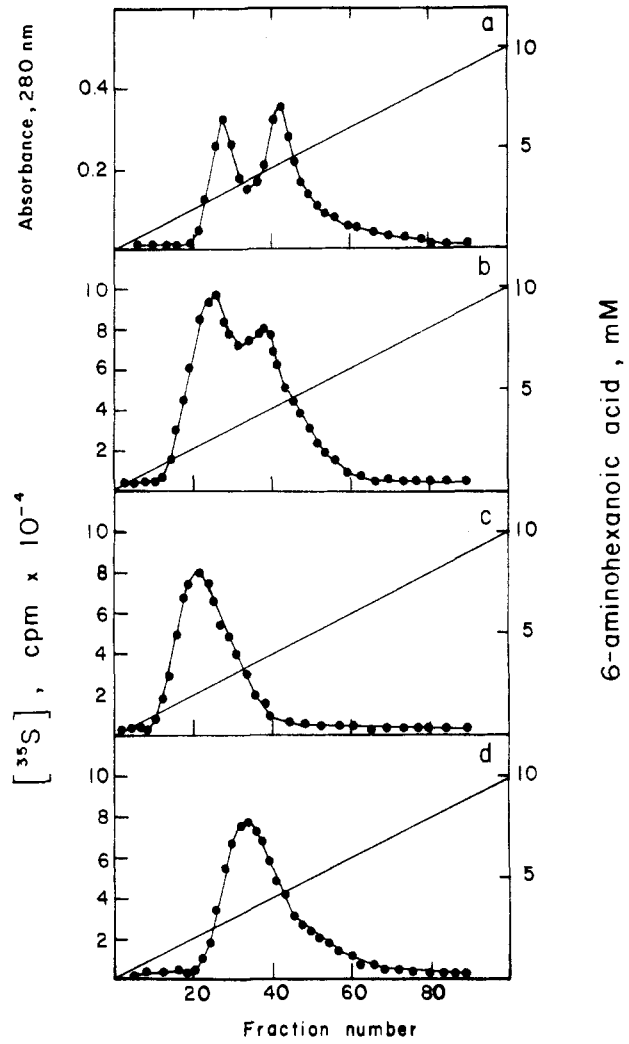

FIGURE 4: L-Lysine-substituted Sepharose affinity chromatography of in vitro synthesized plasminogen. 50 μg of mRNA was translated in a 500-μL reaction mixture containing [^{35}S]methionine as the labeled amino acid. After translation, the reaction mixture was applied on top of a L-lysine-substituted Sepharose column (1.5 × 8 cm) and the column washed with 0.2 M sodium phosphate buffer, pH 7.5. The material adsorbed was eluted with a linear gradient of 6-aminohexanoic acid. Fractions of 1 mL were collected: (a) native monkey plasminogen forms purified from plasma; (b) plasminogen synthesized with mRNA after oligo(dT)–cellulose chromatography; (c) plasminogen synthesized with 23S mRNA purified on Sepharose 4B (Figure 3b); (d) plasminogen synthesized with 18S mRNA purified on Sepharose 4B (Figure 3c).

munoprecipitates after chromatography on concanavalin A–Sepharose, revealing the presence of plasminogen with a size similar to native plasminogen and the disappearance of precursor forms. These results indicate that in the presence of microsomal membranes, the do novo synthesized plasminogen has been segregated into the microsomes and also glycosylated. The segregated plasminogen forms were then compared on their binding properties to L-lysine-substituted Sepharose with those of native monkey plasminogen. Figure 8a shows the two major native monkey plasminogen forms as eluted from the column with a gradient of 6-aminohexanoic acid. The material adsorbed after synthesis with 23S mRNA in the absence and presence of tunicamycin, an inhibitor of *N*-asparagine-linked glycosylation which affects only plasminogen 1, revealed peaks which elute in the same position as the native plasminogen form 1, as shown in Figure 8b. The material adsorbed after synthesis with 18S mRNA under the same conditions revealed peaks which eluted in the same position as the native plasminogen form 2, as shown in Figure 8c.

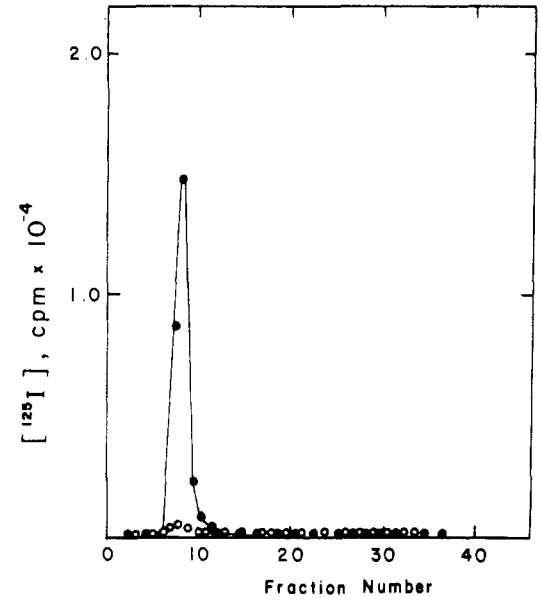

FIGURE 5: Affinity chromatography of the in vitro synthesized plasminogen after reaction with ^{125}I-streptokinase on L-lysine-substituted Sepharose. 50 μg of mRNA was translated in a 500-μL reaction mixture with nonlabeled amino acids. After translation, 0.5 μg of ^{125}I-streptokinase (2.6 × 10^6 cpm/μg) and 10 μg of pancreatic trypsin inhibitor were added to the system, and the mixture was incubated for 30 min at 30 °C and then applied on top of an L-lysine-substituted Sepharose column (1.5 × 3 cm). The column was washed with 0.2 M sodium phosphate buffer, pH 7.4, and the adsorbed material was eluted with 0.2 M 6-aminohexanoic acid. Fractions of 1 mL were collected. Elution profile after translation with total liver mRNA (●); elution profile after incubation in the absence of mRNA from liver (○).

Discussion

The combination of affinity chromatography on L-lysine-substituted Sepharose and immunoprecipitation has made possible the identification of plasminogen as one of the products of the in vitro synthesis directed by mRNA isolated from monkey liver in a cell-free system. Our evidence indicates that the mRNA purified by phenol extraction, affinity chromatography on oligo(dT)–cellulose, fractionation on sucrose gradients, and chromatography on Sepharose 4B columns is active for the synthesis of plasminogen.

The in vitro synthesized plasminogen has many of the physicochemical properties of native plasminogen, as revealed by its capacity to bind to L-lysine-substituted Sepharose (Figure 4) and its reaction with streptokinase (Figure 5). The plasminogen synthesized in the absence of microsomal membranes is nonglycosylated. It has been suggested that the 6-aminohexanoic acid binding difference between the two major plasminogen forms is a consequence of the different content of carbohydrate in these forms (Powell et al., 1981). Our results indicate that these two forms are the result of a synthesis directed by separate mRNAs (Figures 1 and 3). It is known that each major plasminogen form can be separated into about six subforms with different isoelectric points, by isoelectric focusing (Wallén & Wiman, 1970; Summaria et al., 1972). The removal of all of the sialic acid from each isozyme leads to a loss of resolution of two to three of the isoelectric subforms in the isoelectric focusing separation (Siefring & Castellino, 1974a,b) but preserves the 6-aminohexanoic acid binding differences between the two major plasminogen forms. The carbohydrate composition of monkey plasminogen has not been determined; however, the binding of monkey plasminogen form 1 to concanavalin A–Sepharose (Figure 6a) suggests a certain identity between the monkey

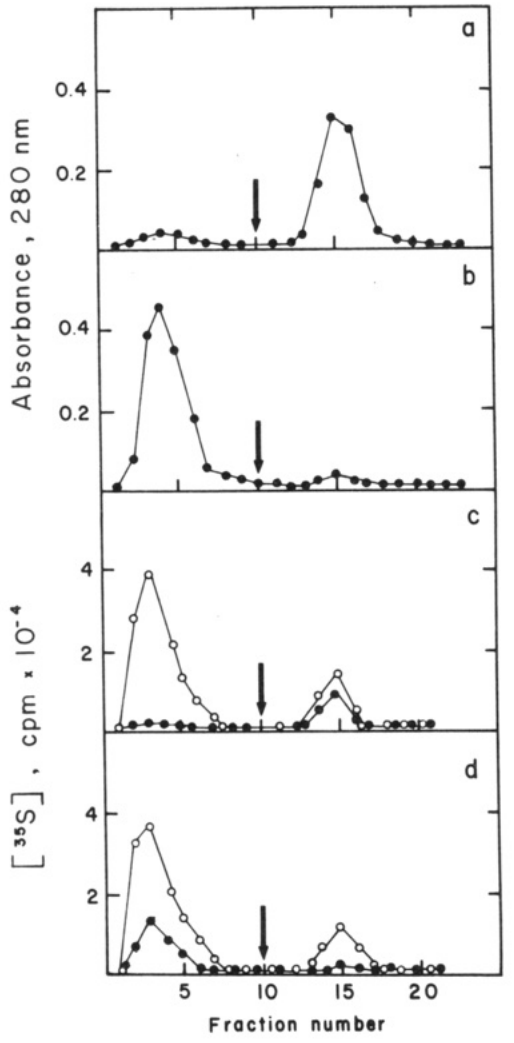

FIGURE 6: Concanavalin A–Sepharose chromatography of the proteins segregated inside dog pancreas microsomes after synthesis directed by mRNA fractions purified from monkey liver. Of each mRNA fraction, 20 μg was translated as described under Materials and Methods. After translation, the microsomal membranes were separated from the reaction mixture, lysed with 0.2% Triton X-100, and then applied on top of a concanavalin A–Sepharose column (1 × 4 cm). The adsorbed proteins were eluted with 0.2 M methyl α-mannoside in phosphate-buffered saline, pH 7.5 (arrow). Fractions of 0.5 mL were collected, and aliquots of 50 μL were used to measure total radioactivity. The rest of the samples were subjected to immunoprecipitation with antibodies to monkey plasminogen. (a) Native monkey plasminogen form 1 from L-lysine-substituted Sepharose; (b) native monkey plasminogen form 2 from L-lysine-substituted Sepharose; (c) ^{35}S-plasminogen synthesized with 23S mRNA; (d) ^{35}S-plasminogen synthesized with 18S mRNA; (○) total ^{35}S radioactivity of unbound and bound proteins; (●) ^{35}S-plasminogen immunoprecipitated with anti-monkey plasminogen antibodies.

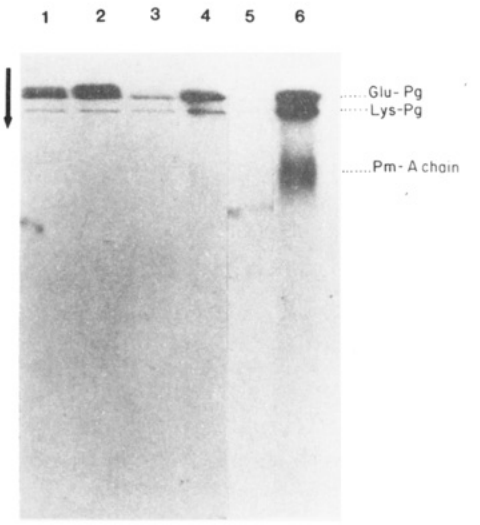

FIGURE 7: SDS–polyacrylamide gel electrophoresis of plasminogen after adsorption to concanavalin A–Sepharose. Lane 1, plasminogen synthesized with 23S mRNA in the absence of dog pancreas microsomes; lane 2, plasminogen synthesized with 18S mRNA in the absence of dog pancreas microsomes; lane 3, plasminogen immunoprecipitated after synthesis with 23S mRNA, segregated inside the dog pancreas microsomes and adsorbed to concanavalin A–Sepharose, as seen in Figure 6a; lane 4, plasminogen immunoprecipitated after synthesis with 18S mRNA, segregated inside the dog pancreas microsomes and not adsorbed to concanavalin A–Sepharose, as seen in Figure 6b; lane 5, immunoprecipitate of the lysed microsomes with no mRNA added to the translation mixture; lane 6, mixture of monkey ^{125}I-Glu-plasminogen, Lys-plasminogen, and plasmin.

and human plasminogen form 1 carbohydrate chains. A similar identity with the carbohydrate chain of human plasminogen form 2 is also suggested for the monkey plasminogen form 2, which as in the human case does not bind to concanavalin A–Sepharose (Figure 6b). This differential binding to concanavalin A was used to identify the in vitro synthesized and glycosylated plasminogen forms. The product of synthesis with 23S mRNA has been identified as the plasminogen form 1 after chromatography on concanavalin A–Sepharose (Figure 6c). Also, the product of synthesis with 18S mRNA corresponds to plasminogen form 2 (Figure 6d). Our results also indicate that the binding of the synthesized and segregated plasminogen form 1 to L-lysine-substituted Sepharose is not affected either when the product is isolated as a glycosylated form or when the synthesis is performed in the presence of tunicamycin, which inhibits the synthesis of the Asn_{288}-linked carbohydrate chain, thereby preventing its binding to concanavalin A–Sepharose (Figure 8b). The synthesized and segregated plasminogen form 2 also presents similar binding properties to L-lysine-substituted Sepharose when compared with native plasminogen form 2 (Figure 8c). These results indicate that the elution pattern of plasminogen forms 1 and 2 from L-lysine-substituted Sepharose is due to differences in the molecules determined early during their synthesis, as seen clearly in Figure 4 where the synthesis is performed in the absence of microsomes and the plasminogen forms already have this property. Therefore, secondary glycosylation which occurs inside the microsomes should be responsible only for the multiplicity of isoelectric subforms, but the synthesis of the two major forms is regulated at the transcriptional level as revealed by the presence in liver of two different mRNAs which code for plasminogen.

Recent studies (Powell & Castellino, 1983) have determined that a peptide isolated from plasminogen form 1 which contains a complex-type Asn_{288}-linked oligosaccharide has an amino acid sequence identical with the corresponding plasminogen form 2 peptide which did not contain oligosaccharide. Therefore, the basis for the lack of oligosaccharide in plasminogen form 2 does not reside in substitution of amino acid residues in the region of the Asn_{288}-linked glycosylation site. The primary structure of human plasminogen has been determined in mixtures containing both forms (Sottrup-Jensen et al., 1978), but similar primary structures of forms 1 and 2 have not been rigorously proven. The presence of separate mRNAs for the two monkey plasminogen forms suggests that their primary structures may be different, although at present we do not have any information about their individual primary structures to substantiate this claim. Perlman et al. (1982) have demonstrated that there are two distinct mRNAs which code for the nonglycosylated cytoplasmic form of yeast in-

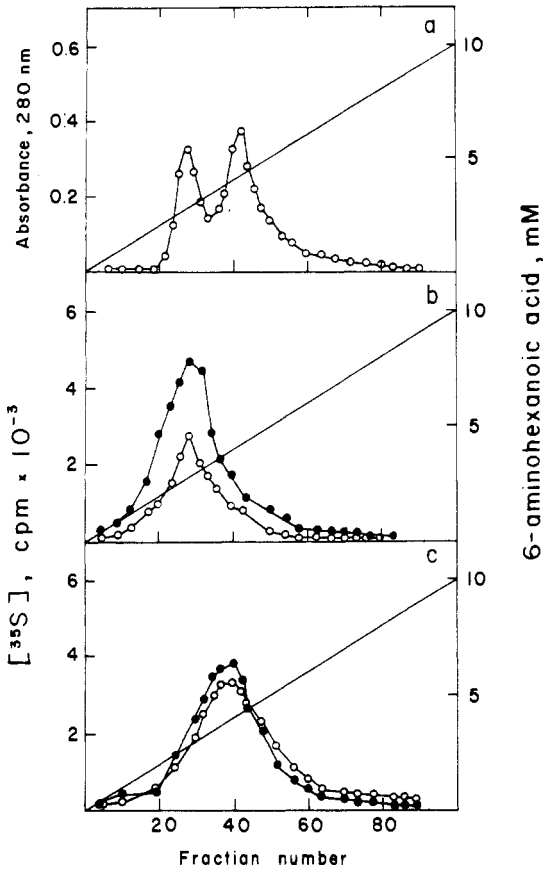

FIGURE 8: L-Lysine-substituted Sepharose affinity chromatography of in vitro synthesized and segregated plasminogen forms. Of each mRNA fraction, 50 μg was translated in the presence of microsomal membranes as described under Materials and Methods. After translation, the segregated proteins were chromatographed on concanavalin A–Sepharose as described in the legend to Figure 6 and then adsorbed to L-lysine-substituted Sepharose (1.5 × 8 cm). The plasminogen was eluted with a linear gradient of 6-aminohexanoic acid. Fractions of 1 mL were collected: (a) native monkey plasminogen forms purified from plasma; (b) ^{35}S-plasminogen synthesized with 23S mRNA in the absence (○) and in the presence (●) of 10 μg/mL tunicamycin; (c) ^{35}S-plasminogen synthesized with 18S mRNA in the absence (○) and in the presence (●) of 10 μg/mL tunicamycin.

vertase and the precursor of the glycosylated secreted invertase. The amino acid sequence and peptide map analysis of these molecules are remarkably similar. However, the signal peptide sequence is significantly different. It has been demonstrated that the synthesis of albumin is regulated either in isolated hepatocytes or in a cell-free system by a feedback mechanism with its propeptide as inhibitor (Weigand et al., 1982). Plasminogen form 1 is released into plasma at approximately half the rate of plasminogen form 2 release (Siefring & Castellino, 1974b). The size of the mRNA for plasminogen form 1 is much bigger than expected for a protein with 790 amino acids, and the resulting precursor form (Figure 7) does not look much bigger than either the plasminogen form 2 precursor (18 S) or the native plasminogen. Whether the synthesis of plasminogen is regulated by a mechanism similar to the one for albumin remains to be determined.

The purification of the mRNAs for the two major plasminogen forms will allow us to study the regulation of the synthesis for each form separately. We have already reconstituted an in vitro heterologous system in which we can follow the segregation and glycosylation of the plasminogen molecule. Further studies with this system will help us to understand better the processes by which the synthesis and secretion of plasminogen are regulated.

Registry No. Plasminogen, 9001-91-6.

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