CELL FREE SYNTHESIS OF HUMAN PROTHROMBIN : IMMUNOLOGICAL CHARACTERIZATION OF THE TRANSLATION PRODUCT

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SUMMARY

mRNAs prepared from adult and fetal human liver were translated in a cell free reticulocyte lysate system. The early precursor of human prothrombin, so called preprothrombin, was purified by immunoaffinity microchromatography. The precursor was identified by immunological competition as a single band displaying in Sodium dodecyl sulfate gel electrophoresis a Mr slightly lower than the Mr of human prothrombin (72 000 instead of 76000). Immunological studies showed that human preprothrombin reacted more efficiently with antibodies raised against denatured prothrombin than with anti plasmatic prothrombin antibodies. The rate of synthesis of the precursor obtained under the direction of adult liver RNA was ten fold higher than that obtained under the direction of fetal liver RNA.

INTRODUCTION

Prothrombin is the coagulation protease zymogen precursor of thrombin (1). It is a plasmatic glycoprotein of 72 000 daltons synthesized in the liver and consisting in a single polypeptide chain with 11 % carbohydrate (2). As many other secretory glycoproteins, human prothrombin is probably synthesized as a precursor form called preprothrombin with an NH_2- terminal extension or signal peptide (3). Partial sequence analysis of the NH_2 terminus of bovine preprothrombin has been reported (4). Evidence is accumulating to indicate that for most of the secretory proteins the proteolytic cleavage of the signal peptide as well as the initial glycosylation of the polypeptide chain occur as cotranslational events prior to the release of the completed protein from the ribosome. Furthermore in the case of prothrombin, the newly synthesized precursor is submitted to an additional specific processing event : the vitamin K-dependent carboxylation of ten specific glutamic acid residues in the NH_2- terminal region of the protein (5). The resulting ten γ carboxyglutamic acid residues

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enable prothrombin to bind calcium ions and to be normally converted into thrombin (2).

In order to study the different molecular events involved in the post-translational processing of human prothrombin in the liver, it is necessary in a first stage to synthesize and characterize in a cell free system, preprothrombin, the early precursor of plasmatic prothrombin.

This paper describes for the first time cell free synthesis of human prothrombin ; the neosynthesized protein is recognized by antibodies directed against denatured plasmatic prothrombin but only scarcely by antibodies directed against the active form of prothrombin. The amount of preprothrombin neosynthesized under the direction of fetal liver RNA is ten fold lower than the amount obtained when adult liver RNA is used.

MATERIAL AND METHODS

Material : Human adult liver was obtained after surgical partial hepatectomy ; fetal liver was obtained from therapeutic abortion. ^{35}S- methionine was purchased from Amersham. Glutaraldehyde- activated Ultrogel was supplied by Industrie Biologique Française ; acrylamide, bisacrylamide, Royal X-Omatic R_1 films by Eastman Kodak ; ^{14}C markers, Econofluor, Prosotol, Enhance autoradiography enhancer by New England Nuclear, tRNA from calf liver by Boeringher, methylmercury hydroxyde by Ventron Alpha.

Methods : Human prothrombin was purified according to Mann (6) and ^{14}C labeled according to Dottavio-Martin and Ravel (7).

Immunological procedures : Two kinds of antibodies were used, raised against non-denatured and denatured plasmatic prothrombin. Prothrombin was denatured by heating in the presence of 1 % (w/v) Sodium dodecyl sulfate and 2 % (v/v) β mercaptoethanol for 3 min at 100 °C. Then the denatured preparation was diluted ten fold in 0.15 M NaCl buffered with 10 mM sodium phosphate (pH 7.4) before being injected to rabbits by intradermal injections (8). The antibodies raised against non-denatured prothrombin were purified on an prothrombin-bound ultrogel column (9). Ultrogel-bound prothrombin was first denatured by extensive washing with 50 mM Tris-HCl pH 8 buffer containing 2 % (w/v) Sodium dodecyl sulfate and 2 % (v/v) β mercaptoethanol at room temperature. The column was then equilibrated with 0.1 M phosphate buffer pH 7.4 containing NaCl 0.15 M, 0.5 % (v/v) Triton and 0.5 % (w/v) sodium desoxycholate. Rabbit antiserum raised against denatured prothrombin was passed through the column and the specific antibodies were purified as above. Both antibodies were tested by double immunodiffusion in agarose gel and bound to glutaraldehyde activated ultrogel (9).

Isolation of RNA : Total cellular RNA from adult and fetal human liver were isolated by a method of ethanol precipitation in guanidine-HCl slightly modified from (10, 11, 12) as already described elsewhere (9). Some further modifications were brought to this technic : the first ethanol precipitation was reduced to 30 minutes at - 20°C instead of overnight. 0.5 % (w/v) lauroyl

sarcosine was added to all the solutions until the last two steps of washing in 66 % (v/v) then 95 % (v/v) ethanol. Total cellular high molecular weight RNA was used to direct protein synthesis without further fractionation.

Cell free synthesis and analysis of the translation products : Total liver RNA first denatured for 5 min at room temperature in 5 mM methylmercury hydroxyde were translated in a microccocal nuclease-treated rabbit reticulocyte lysate system according to the procedure of Pelham and Jackson (13) in the presence of calf liver tRNA (40 μg/ml), the final concentration of methylmercury hydroxyde being 0.2 mM. Neosynthesized products were purified by a micromethod of immunoaffinity chromatography using microcolumns consisting of disposable Eppendorf blue tips for automatic pipets containing 5 μl of antibodies-bound resins, as described in (9). The microcolumns were washed thouroughly and subsequent elution was performed at 37°C using 0.2 % (w/v) dodecyl sulfate, 1 % (v/v) β mercaptoethanol in 10 % (v/v) acetic acid.

Translation products were analyzed by Sodium dodecyl sulfate- polyacrylamide gradient gel electrophoresis according to Laemmli (14). The gels were dried and neosynthesized proteins revealed by fluorography using preflashed Kodak XRI films. Neosynthesized preprothrombin was identified by immunological competition using an antiprothrombin column previously saturated with an excess unlabeled prothrombin.

RESULTS

Immunoreactivity of the antibodies raised against plasmatic prothrombin was compared to the reactivity of the anti denatured prothrombin antibodies. In fig. 1, it can be seen that there is no detectable cross reativity between non-denatured and denatured prothrombin with respect to both antibodies.

In fig. 2 is shown the pattern of total polypeptides neosynthesized under the direction of liver RNA. Fig. 3 shows the translation products specifically retained by immunoabsorbent microcolumn using antibodies raised against either non-denatured (Fig. 3.4) or denatured prothrombin (Fig. 3.3.) It is clear that anti denatured prothrombin antibodies retained more efficiently neosynthesized polypeptides than antiprothrombin antibodies. In the eluate from anti denatured prothrombin column, only one band was observed (Mr = 72 000 as compared to 76 000 for ^{14}C plasmatic prothrombin) without detectable contaminant ; by contrast material eluted from anti non-denatured protein column exhibited two faint bands : a major one with the same Mr as the polypeptide retained by anti denatured prothrombin antibodies, and a minor one with a slightly lower Mr (69 000) probably due to a proteolytic cleavage of the major polypeptide. Identification of these polypeptides as preprothrombin was confirmed by immuno-

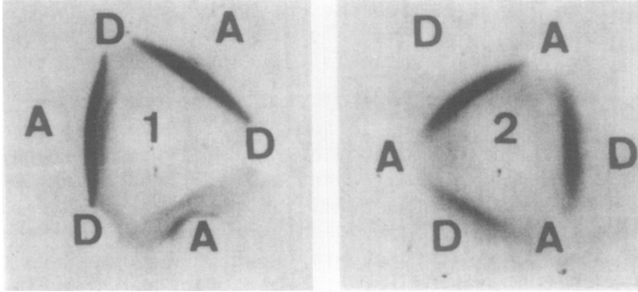

Figure 1. Double immunodiffusion analysis of antisera raised against denatured and non-denatured prothrombin. A : Non-denatured prothrombin, each well containing 10, 5 and 2,5 μg respectively ; D : prothrombin denatured by heating at 100° for 3 min in the presence of 1 % (w/v) Sodium dodecyl sulfate and 1 % (v/v) β mercaptoethanol. The wells contained the same amount of protein as for "A".

1. Purified anti non-denatured prothrombin antibodies (20 μg).
2. Purified anti denatured prothrombin antibody (20 μg). Staining with amido-black.

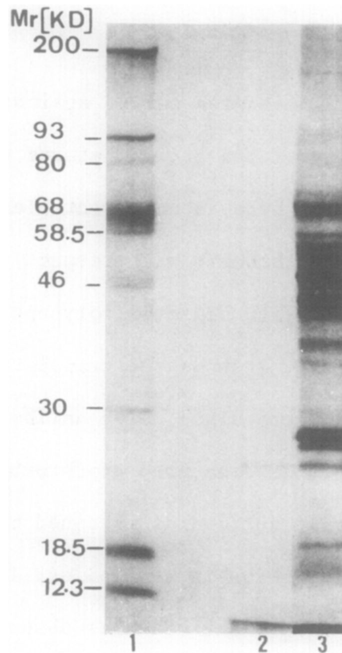

Figure 2. Sodium dodecyl sulfate polyacrylamide gradient gel analysis of the polypeptides synthesized under the direction of human liver RNAs.

1. ^{14}C labeled protein markers : myosin heavy chain (Mr = 200 kilo daltons) ; muscle phophosrylase (Mr = 93 KD) ; muscle phosphofructokinase (Mr = 80 KD) ; bovine serum albumin (Mr = 68 KD) ; glucose phosphate isomerase (Mr = 58.5 KD) ; ovalbumin (Mr = 46 KD) ; carbonic anhydrase (Mr = 30 KD) ; lactoglobulin A (Mr = 18.5 KD) ; cytochrome (Mr = 12.3 KD).
2. Blank without addition of exogenous RNA.
3. Addition of 5 μg human liver RNA per 25 μg total translation mixture. 10 μl of this mixture were denatured by Sodium dodecyl sulfate , then applied to the gel.
   Labeling with ^{3}H leucine ; revelation by fluorography.

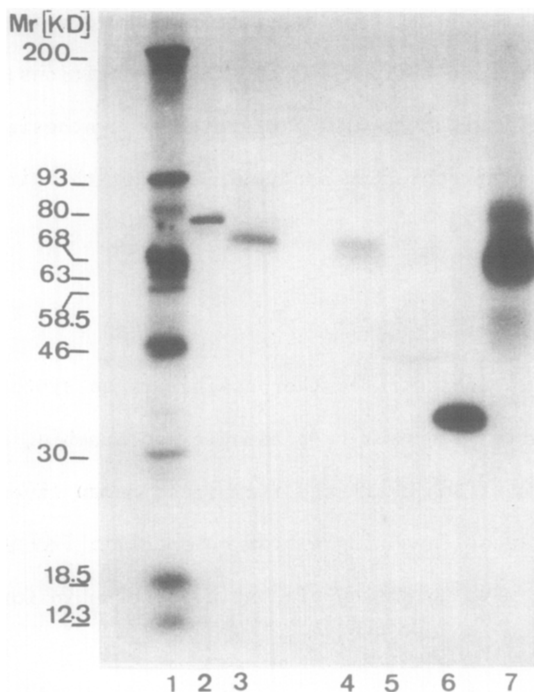

Figure 3. Purification by immunoaffinity microchromatography of neosynthesized liver proteins. 65 µl of translation mixture obtained under the direction of liver RNA, with ^{35}S methionine as labeled residue, were applied to a combinaison of microcolumns containing, from the top to the bottom, 10 µl non immune Ig G resin, 5 µl anti serum albumin resin, 5 µl anti aldolase B resin, 5 µl anti non-denatured prothrombin resin, then 5 µl anti denatured prothrombin resin.

1. ^{14}C labeled protein markers (idem Fig. 2, except that L' pyruvate kinase (Mr = 63 KD) is represented here ;
2. ^{14}C labeled human plasmatic prothrombin ;
3. Eluate from the anti denatured prothrombin column ;
4. Eluate from the anti non-denatured prothrombin column ;
5. Eluate from the non immune column ;
6. 1 : 20 of the eluate from the anti aldolase B column ;
7. 1 : 55 of the eluate from the anti serum albumin column.

Revelation by fluorography.

logical competition : bands in Fig. 3.3 and 3.4 disappeared when immunoabsorbent microcolumns were first saturated with an excess of unlabeled prothrombin (not shown here).

Quantitative estimations performed either prior to or after electrophoresis of the translation products eluted from immunoabsorbent microcolumns, show that purified preprothrombin represented about 0.1 % of total neosynthesized proteins. This figure can be compared to serum albumin wich was found, in the same conditions, to represent about 8 % of the total synthesized pro-

teins. Cell free synthesis experiments were performed using human fetal liver RNA in place of human adult liver RNA. In these conditions, neosynthesized preprothrombin represents only 0.01 % of total neosynthesized proteins, i.e. 10 fold lower than preprothrombin synthesized under the direction of adult liver RNA.

DISCUSSION

The present work demonstrates the possibility to synthesize human preprothrombin, the early precursor of plasmatic prothrombin, in a cell free system starting from total RNA extracted from fresh human liver tissue. Preprothrombin can be isolated in a single step on specific immunoabsorbent microcolumn and competes with unlabeled plasmatic prothrombin for the binding to the immunoabsorbent.

Human preprothrombin displays a Mr slightly lower than that of plasmatic prothrombin. This difference is accounted for by the absence of glycosylation. Moreover the highly probable existence of a signal peptide(which is expected to represent from 2000 to 2500 daltons) (4) is masked by the absence of glucidic moiety in preprothrombin. Such a signal peptide has been recently found and partially sequenced by Mac Gillivray et al in bovine preprothrombin (4). From comparison between the results obtained with both antiprothrombin antibodies used in this study, it follows that preprothrombin is more easily recognized by anti denatured prothrombin antibodies than by antibodies raised against plasmatic prothrombin. Similar data have been recently reported for other secretory proteins or polypeptidic hormones synthesized in cell free systems and essentially recognized by anti denatured protein antibodies (15). The difference of antigenicity between preprothrombin and mature prothrombin cannot be ascribed to the own antigenicity of the glucidic moiety of the molecule, since denatured antigen is glycosylated as well as active prothrombin. It is more probable that conformation of the neosynthesized polypeptidic chain is more closely related to the denatured prothrombin tertiary structure than to

that of active plasmatic prothrombin. Nevertheless, it is noticeable that preprothrombin can be recognized by both antibodies whereas there is no cross reaction between these antibodies with respect to non-denatured and denatured prothrombin. This suggests that the actual conformation of preprothrombin is unique as compared to both antigens used to prepare the antisera.

It could mean that different steps in the processing of human prothrombin, especially glycosylation and carboxylation, play an important role in the completion of the tertiary structure of the plasmatic protein. So, quantitative estimations of the amount of neosynthesized human preprothrombin are difficult and probably always underestimated. Our evaluation of the amount of preprothrombin neosynthesized (0.1 % of total protein synthesis is much less than that reported by Gillivray et al (1 %) in the case of bovine preprothrombin using an analogous cell free system (16). In the absence of any report by these authors of the degree of homogeneity of preprothrombin in the immunoprecipitate, estimated by gel electrophoresis, these results cannot be accurately compared. In contrast, the amount (0.1 %) of human preprothrombin neosynthesized under the direction of adult liver RNA can be interestingly compared to that obtained under the direction of fetal liver RNA (0.01 %). Such results should be related to the concentration of human prothrombin in plasma which is about five fold lower in fetal than in adult (17). From our data it could be presumed that the differences observed in the plasmatic concentration of human prothrombin in fetal and adult are related to differences in the concentrations of translatable prothrombin mRNA in fetal and adult liver.

In conclusion, we have been able to synthesize preprothrombin in a cell free system. Immunological studies showed that the structure of the precursor is very different from that of plasmatic mature prothrombin and close but not identical to that of denatured prothrombin. Finally, the rate of synthesis of human preprothrombin under the direction of adult liver RNA is ten fold higher than that obtained using fetal liver RNA.

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