Synthesis of α2-macroglobulin in rat hepatocytes and in a cell-free system

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Received 22 November 1982

The biosynthesis and secretion of α2-macroglobulin was studied in rat hepatocyte primary cultures. After immunoprecipitation of α2-macroglobulin from a cell homogenate and the hepatocyte medium, two forms of α2-macroglobulin with app. M_r of 176000 and 182000, respectively, were identified. A precursor–product relationship for the two α2-macroglobulin forms was demonstrated by a pulse-chase experiment. The cellular form of α2-macroglobulin could be deglycosylated by endoglucosaminidase H, whereas the medium form of α2-macroglobulin remained unaffected. On the other hand, only the medium form of α2-macroglobulin was found to be susceptible to neuraminidase. In vitro translation of rat liver poly(A)$^+$ RNA resulted in a translation product of an app. M_r of 162000.

α2-Macroglobulin *Rat hepatocyte* *Deglycosylation* *In vitro translation*

1. INTRODUCTION

α2-Macroglobulin is an acute phase protein in the rat. Its biosynthesis increases markedly after inflammatory stimuli [1,2]. It is believed that α2-macroglobulin is synthesized in the liver and secreted into the blood [3,4], where it functions as an inhibitor of plasma endopeptidases. Unlike most other protein inhibitors of proteinases α2-macroglobulin inhibits by entrapping the proteinase molecules through a conformational change in the inhibitor [5]. Rat α2-macroglobulin is thought to consist of 4 probably identical subunits of M_r of ~180000–190000 each [5]. It has been found that rat α2-macroglobulin is a glycoprotein containing ~16% of carbohydrate [6–8]. At present little information is available on the mechanism(s) of biosynthesis of α2-macroglobulin.

Here, hepatocytes as well as cell-free systems have been used to learn more about the biosynthesis of α2-macroglobulin. It will be shown that two forms of α2-macroglobulin exist: an intracellular mannose-rich, and an extracellular complex-type glycoprotein. The cell-free translation of full sized α2-macroglobulin will be presented.

2. MATERIALS AND METHODS

L-[^{35}S]Methionine (> 600 Ci/mmol), D-[2-^{3}H]-mannose (13 Ci/mmol) and L-[5,6-^{3}H]fucose (45 Ci/mmol) were purchased from the Radiochemical Centre (Amersham). D-[6-^{3}H]Galactose (15 Ci/mmol) was obtained from New England Nuclear (Boston). Endoglucosaminidase H from *Streptomyces griseus* H12 was purchased from Seikagaku Kogyo (Tokyo). Neuraminidase from *Vibrio cholerae* and tunicamycin were from Calbiochem-Behring (Giessen). Protein A–Sepharose CL-4B was from Pharmacia (Freiburg). The preparation of rat hepatocytes and the labeling conditions with [^{35}S]methionine and the ^{3}H-labeled sugars were described in [9,10]. The experimental details for the immunoprecipitation of α2-macroglobulin, the binding conditions of the α2-macroglobulin antibody complexes to protein A–Sepharose, the conditions for polyacrylamide slab-gel electrophoresis and subsequent fluorography have been described in [9,10].

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Published by Elsevier Biomedical Press
00145793/83/0000—0000/$3.00

2.1. *Treatment of α2-macroglobulin with glycosidases*

The α2-macroglobulin–IgG complexes eluted from the protein A–Sepharose were exhaustively dialyzed against 50 mM phosphate buffer (Na^+) (pH 6.0), containing 0.01% sodium dodecyl sulfate. Incubation of radioactively-labeled α2-macroglobulin obtained from one dish was carried out in 0.1 ml of the same buffer with 5 munits of endoglucosaminidase H at 37°C for 16 h. The incubation with neuraminidase was done in 50 mM sodium acetate buffer (pH 5.5), 0.15 M NaCl and 9 mM $CaCl_2$ at 37°C for 16 h.

2.2. *Cell-free synthesis of α2-macroglobulin*

Male Wistar rats, kindly provided by Professor Dr H. Ueberberg (Thomae GmbH, Biberach), were injected intramuscularly with 0.5 ml turpentine/100 g body weight. The animals were killed 18 h after injection and starvation, and the RNA was extracted with guanidinium HCl from the livers frozen in liquid nitrogen [11]. Oligo(dT)-cellulose chromatography was used for the isolation of poly(A)$^+$ RNA which then was translated into proteins in the presence of a rabbit reticulocyte lysate [12]. RNase inhibitor purified from human placenta [13] was routinely added to the translation mixture of 1 unit/μl.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of α2-macroglobulin by rat hepatocytes*

Hepatocyte primary cultures were pulse-labeled with [^{35}S]methionine for 10 min followed by a chase with unlabeled methionine. At different times ranging from 10–120 min, cells were separated and centrifuged. α2-Macroglobulin was immunoprecipitated from the supernatant obtained from the homogenate or from the medium. It can be seen particularly from lanes 7 and 8 of fig.1 that two forms of α2-macroglobulin, which differ slightly in their electrophoretic mobilities, are present in the hepatocytes, whereas the hepatocyte medium contains only the α2-macroglobulin species with the higher app. M_r. From a plot of the electrophoretic mobilities *vs* log M_r, app. M_r of 182000 and 176000 were estimated for the medium and cellular forms of α2-macroglobulin, respectively. The α2-macroglobulin form – secreted by the hepatocytes – exhibits the same mobility during electrophoresis as the α2-macroglobulin purified from rat plasma. Fig.1 shows that between 60 (lane 6) and 90 min (lane 8) after the chase, α2-macroglobulin appeared in the medium. A precursor–product relationship between the two α2-macroglobulin forms, characterized by different M_r, seems evident from these findings.

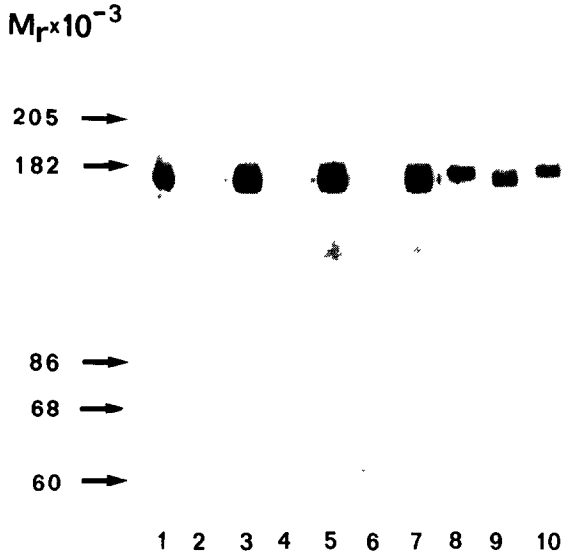

Fig.1. Synthesis and secretion of α2-macroglobulin after pulse-labeling with [^{35}S]methionine. Rat hepatocyte primary cultures were incubated with [^{35}S]methionine (25 μCi/dish) for 10 min followed by a chase with unlabeled methionine at a final concentration of 3 mM. α2-Macroglobulin was immunoprecipitated from the cells for 10 min (lane 1), 30 min (lane 3), 60 min (lane 5), 90 min (lane 7), 120 min (lane 9), and from the medium for 10 min (lane 2), 30 min (lane 4), 60 min (lane 6), 90 min (lane 8) and 120 min (lane 10) after the chase. The following M_r markers were used: myosin from rabbit muscle (205000), rat plasma α2-macroglobulin (182000), conalbumin (86000), bovine serum albumin (68000) and catalase (60000).

Further evidence for the existence of two forms of α2-macroglobulin of different app. M_r was obtained from digestion experiments of α2-macroglobulin from cells and from medium with endoglucosaminidase H and neuraminidase, respectively. Fig.2 shows the results of the endoglucosaminidase H treatment. Whereas the cellular α2-macroglobulin form was susceptible to endo-

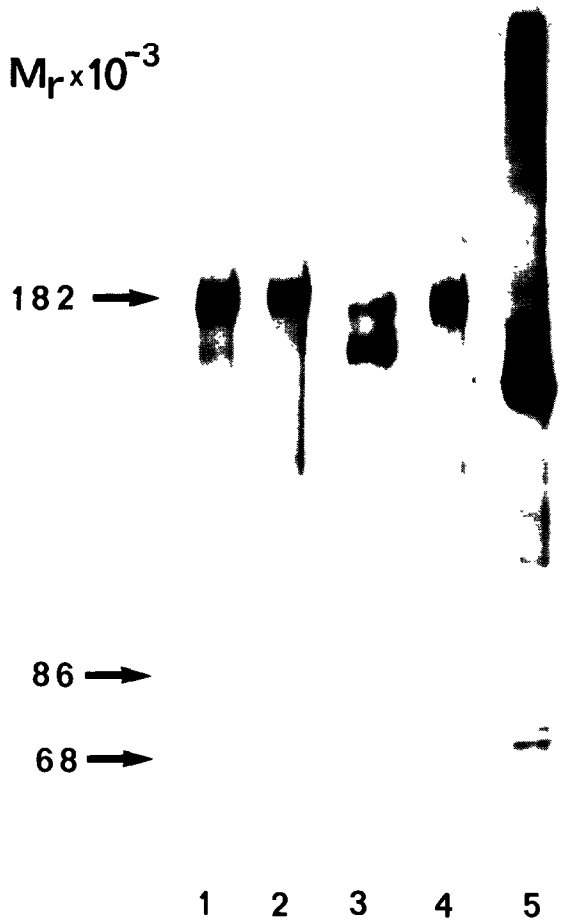

Fig.2. Deglycosylation of α2-macroglobulin with endoglucosaminidase H. Rat hepatocyte primary cultures were incubated in medium containing [^{35}S]methionine (25 μCi/dish). After 2 h α2-macroglobulin was immunoprecipitated from the cells (lanes 1,3) as well as from the medium (lanes 2,4), incubated without (lanes 1,2), or with 5 munits of endoglucosaminidase H (lanes 3,4) at 37°C for 16 h as described in section 2. Tunicamycin was given to the hepatocyte culture medium at 3 μg/ml. After 1 h the medium was changed and medium containing [^{35}S]methionine and tunicamycin at 3 μg/ml was given to the cell cultures. After a labeling time of 2 h, α2-macroglobulin was immunoprecipitated from the cells (lane 5). M_r markers: α2-macroglobulin from rat plasma (182000), cónalbumin (86000), and bovine serum albumin (68000).

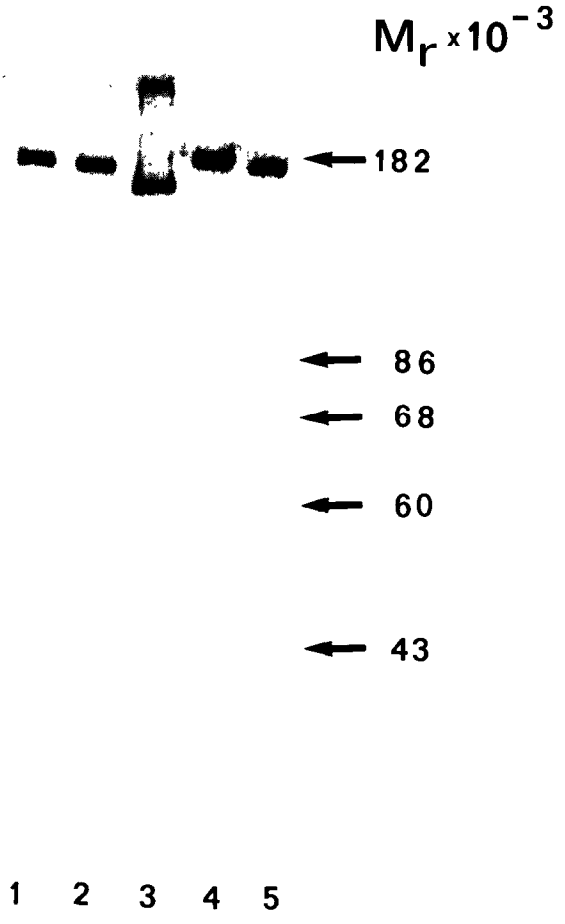

Fig.3. Treatment of α2-macroglobulin with neuraminidase. After a 2-h labeling period with [^{35}S]methionine α2-macroglobulin was immunoprecipitated from the cells (lanes 1,2) and from the medium (lanes 4,5), and incubated without (lanes 1,4), or with (lanes 2,5) 10 munits of neuraminidase from *V. cholerae* at 37°C for 16 h as described in section 2. α2-Macroglobulin immunoprecipitated from hepatocytes after tunicamycin treatment as described in fig.2 (lane 3). M_r markers: α2-macroglobulin from rat plasma (182000), conalbumin (86000), bovine serum albumin (68000), catalase (60000) and ovalbumin (43000).

glucosaminidase H (lane 3), the medium form of α2-macroglobulin was unaffected (lane 4). The α2-macroglobulin polypeptide obtained after endoglucosaminidase H treatment of the cellular α2-macroglobulin exhibited an increased electrophoretic mobility, but different from that of α2-macroglobulin immunoprecipitated from hepatocytes after incubation with tunicamycin (lane 5), which blocks the formation of *N*-aspar-

agine-linked oligosaccharide chains. This difference in M_r might be due to the fact that endoglucosaminidase H leaves the first *N*-acetylglucosamine molecule of each oligosaccharide chain attached to asparagine. As shown in fig.3 the α2-macroglobulin medium form, resistant to the action of endoglucosaminidase H, was susceptible to neuraminidase (lanes 4,5) indicating sialic acid to be a terminal sugar of the oligosaccharide chains. The neuraminidase digestion led to an α2-macroglobulin of slightly higher electrophoretic mobility corresponding to an app. M_r of ~174000. In addition, it can be seen in fig.3 that the faint band above the α2-macroglobulin cellular form (lane 1) – representing the α2-macroglobulin medium form in the cells before secretion – disappeared after digestion with neuraminidase (lane 2).

In further experiments we have studied the incorporation of radioactively-labeled monosaccharides into α2-macroglobulin. We found that [^{3}H]mannose was predominantly incorporated into the intracellular α2-macroglobulin form, whereas [^{3}H]galactose and [^{3}H]fucose were nearly exclusively incorporated into the medium form of α2-macroglobulin. These data will be published elsewhere.

From the findings described, it can be concluded that α2-macroglobulin is first synthesized as a mannose-rich glycoprotein, which then is transformed to a complex-type glycoprotein. Only the latter form is secreted. Similar observations have been made recently in the case of α_1-antitrypsin [9,10].

3.2. *Synthesis of α2-macroglobulin in vitro*

Poly(A)-rich RNA, isolated from livers of turpentine-treated rats by guanidinium HCl extraction, was translated into proteins using a rabbit reticulocyte lysate. The translation products labeled with [^{35}S]methionine were treated with an antiserum against rat α2-macroglobulin. The fluorogram (fig.4) shows that a polypeptide of an app. M_r of 162000 had been synthesized. The yield of the in vitro synthesized α2-macroglobulin can be increased at least 5-fold by the use of RNase inhibitor purified from human placenta [13]. When unlabeled α2-macroglobulin was added in increasing concentrations to the translation mixture (lanes 2–5) a competition was observed. No competition was found with rat serum albumin used as a control (lane 6). Since α2-macroglobulin is a secretory protein, and since, with a few exceptions [14–17], the in vitro synthesized secretory proteins are made as larger M_r precursors with N-terminal extensions, it is likely that α2-macroglobulin is also made as a larger M_r precursor. The comparison with the mature, unglycosylated α2-macroglobulin isolated from tunicamycin-treated hepatocytes did not

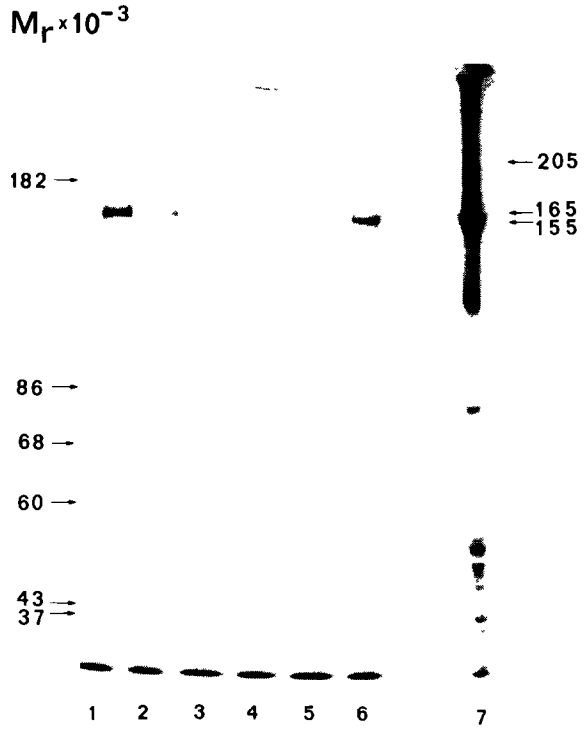

Fig.4. Cell-free synthesis of α2-macroglobulin. Poly(A)-rich RNA from rat liver was translated in a cell-free system in vitro, immunoprecipitated, separated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis and analyzed by autoradiography: lane 1, α2-macroglobulin synthesized in vitro; lanes 2–6, same as lane 1, except that 0.5 μg (lane 2), 1.5 μg (lane 3), 3 μg (lane 4), and 8 μg (lane 5) of rat plasma α2-macroglobulin, and 8 μg of rat serum albumin (lane 6) have been added to the translation mixture before immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein M_r markers were myosin (205000), α2-macroglobulin from rat plasma (182000), β'-subunit (165000), and β-subunit (155000) of RNA polymerase of *E. coli*, conalbumin (86000), bovine serum albumin (68000), catalase (60000), ovalbumin (43000) and alcohol dehydrogenase (37000).

show a difference in electrophoretic mobilities (lane 7). Due to the high M_r of α2-macroglobulin it is clear that our separation system is hardly suited to resolve small M_r differences of 2000–3000, which would be expected for α2-macroglobulin and an α2-macroglobulin precursor carrying a signal peptide of about 20 amino acids. Radioactive Edman degradation is in progress to solve this problem.

ACKNOWLEDGEMENTS

We are greatly indebted to Professor Dr K. Decker, Freiburg, for his continuous interest and support during the course of this work. The authors also like to thank Professor Dr K. Hilse, Freiburg, for his help with the preparation of rabbit reticulocyte lysates. The excellent assistance of H. Gottschalk with the preparation of the manuscript is appreciated. This work has been supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the Müller-Fahnenberg-Stiftung.

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