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BIOSYNTHESIS, INTRACELLULAR PROCESSING AND SECRETION OF HAPTOGLOBIN IN CULTURED RAT HEPATOCYTES

Yoshio Misumi, Yukari Tanaka and Yukio Ikehara*

Department of Biochemistry, Fukuoka University School of Medicine
Nanakuma, Jonan-ku, Fukuoka 814-01, Japan

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SUMMARY : Biosynthesis, intracellular processing and secretion of the hetero-tetrameric ($\alpha_2\beta_2$) glycoprotein, haptoglobin, were studied in primary cultured rat hepatocytes. The results obtained from pulse-chase experiments demonstrated that haptoglobin was initially synthesized as a larger precursor (pro-form), a single polypeptide chain comprising both the α- and β-subunits, and immediately cleaved into subunits during intracellular transport, although about 8% of the newly synthesized haptoglobin was secreted as a pro-form. Monensin which impedes the secretory process at the Golgi complex blocked the complete glycosylation of β-subunit but rather accelarated the conversion of the pro-form to subunits. These results indicate that the proteolytic processing of the haptoglobin precursor takes place at an early stage before the Golgi complex of the intracellular transport.

Most secretory proteins are initially synthesized as larger precursors (pre-forms) with an extended NH_2-terminal sequence which is co-translationally cleaved (1). In some cases of secretory proteins such as albumin (2,3), insulin (4) and parathormone (5), the newly synthesized proteins exist intracellularly as an another type of precursors (pro-forms), which are processed to their final mature forms by post-translational proteolysis prior to secretion. Such proteolytic processing has been shown to occur at a late stage of the secretory process, usually within the Golgi complex and related vesicles(2-7)

Haptoglobin is a plasma glycoprotein which is synthesized and secreted by hepatocytes (8). Its function is to form a strong and stable complex with hemoglobin which has been released from erythrocytes and foster the recycling of heme iron (8,9). Haptoglobin is a tetrameric protein composed of two types of polypeptide chains, α and β, which are covalently associated by disulfide

* To whom correspondence should be addressed.

Abbreviations used: SDS, sodium dodecyl sulfate; Endo H, endo-β-N-acetyl-glucosaminidase H; MEM, minimum essential medium; PBS, phosphate buffered saline; Kd, kilo daltons.

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bonds, denoted as "βααβ". Molecular weights of α- and β-subunits of rat haptoglobin are estimated to be 9.5Kd and 35Kd, respectively (8,10) and only the β-subunit is glycosylated (8). Recently Haugen et al.(10) reported that the primary in vitro translation product of rat haptoglobin mRNA was a single polypeptide that contained the elements of both the α- and β-subunits of the protein. They suggested the possibility that haptoglobin is synthesized in vivo as a single precursor protein that is proteolytically processed post-translationally to form the dissimilar α- and β-subunits of the native protein (10).

To explore this possibility we examined the biosynthesis, intracellular processing and secretion of haptoglobin in cultured rat hepatocytes, demonstrating that the protein was in fact initially synthesized as a larger precursor, which was immediately processed to the subunits. As opposed to the results obtained with proalbumin (11) and pro-opiomelanocortin (12), the intracellular proteolytic processing of the haptoglobin pro-form was not blocked by the carboxylic ionophore monensin (13), indicating that its processing occurs at an early stage of transport.

MATERIALS AND METHODS

Materials. L-[^{35}S]Methionine (1,200 Ci/mmol) and EN^{3}HANCE were purchased from New England Nuclear, Boston, MA; monensin from Calbiochem-Behring Corp., La Jolla, CA. Rabbit hemoglobin, tunicamycin and protein A-Sepharose were obtained from Sigma Chemicals, St. Louis, MO; collagenase (type I) from Worthington Biochem. Corp., Freehold, NJ; endo-β-N-acetylglucosaminidase (Endo H) from Seikagaku Kogyo, Tokyo; Eagle's minimum essential medium (MEM) from Nissui Seiyaku Co., Tokyo.

Preparation of Rat Haptoglobin and Its Antiserum. Rat haptoglobin was purified from the plasma of turpentine-treated (48 hr) rats by affinity chromatography, taking advantage of the high affinity specific interaction of haptoglobin with hemoglobin (10,14). Rabbit hemoglobin (100 mg) was coupled with BrCN-activated Sepharose 4B (20 ml) according to Trayer and Hill (15,16). Rat plasma (20 ml) was diluted with an equal volume of phosphate buffered saline (PBS), pH 7.4, and applied to a hemoglobin-Sepharose column (1.8 x 8 cm) which had been equilibrated with the same buffer. The column was washed with 200 ml of PBS and then with 1,000 ml of 0.5 M NaCl in 10 mM Na-phosphate buffer, pH 7.4. Haptoglobin was then eluted from the column with 6 M guanidine hydrochloride. Fractions of the protein peak were immediately pooled and extensively dialyzed against 20 mM Tris-HCl-50 mM NaCl, pH 7.5. SDS-gel electrophoresis revealed that the preparation contained α- and β-subunits of haptoglobin and trace amounts of rabbit hemoglobin which was co-eluted from the affinity column. The yield of haptoglobin was usually about 60 mg under the above conditions. Antibodies against rat haptoglobin were raised in rabbits as described previously (17). Ouchterlony immunodiffusion and immunoelectrophoresis demonstrated that all the antisera obtained were monospecific for rat haptoglobin.

Hepatocyte Culture. Hepatocytes were isolated from adult male Wistar rats, weighing 200-250 g, by the collagenase perfusing method of Seglen (18). Isolated rat hepatocytes were cultured as described previously (11,19).

Pulse-Chase Experiments. Cells were preincubated at 37°C for 30 min with or without monensin (1×10^{-6} M) in Eagle's MEM (20). After two washings with Dulbecco's PBS (20), cells were pulse-labeled for 10 min with [^{35}S]methionine (100 µCi/1.5 ml/dish) in the medium lacking cold methionine. After two washings with Dulbecco's PBS, the cells were cultured in 2 ml of Eagle's MEM. At the indicated times, the cells and medium were taken for immunoprecipitation of haptoglobin. Monensin, when indicated, was present in the medium throughout the pulse-chase periods.

Immunoprecipitation and Endoglycosidase Treatment. Cell lysates and medium were prepared as described previously (11,19). Immunoprecipitates of [^{35}S]-labeled haptoglobin were prepared from each samples as previously described for albumin and α_1-protease inhibitor (11,19). Treatment of the newly synthesized haptoglobin with Endo H (final concentration, 0.2 unit/ml) was carried out for 16 hr at 37°C after the samples were adjusted to 0.05 M citrate buffer, pH 5.5 (19).

Polyacrylamide Gel Electrophoresis. SDS-gel electrophoresis was performed on 12.5% polyacrylamide slab gels (21). After electrophoresis, gels were fixed with 30% methanol-10% trichloroacetic acid-7% acetic acid for 1 hr and treated with EN^{3}HANCE, followed by drying and fluorography (22). Apparent molecular weights were determined by co-electrophoresis of marker proteins; rat serum albumin (68,000), α_1-protease inhibitor (56,000), ovalbumin (45,000), trypsin (23,000) and α_{2u}-globulin (20,000).

RESULTS AND DISCUSSION

When hepatocytes were pulse-labeled with [^{35}S]methionine and chased, secretion of the labeled haptoglobin started at 10 min as observed for albumin (11) and α_1-protease inhibitor (19), and reached about 75% of the total labeled haptoglobin by 3 hr of chase (Fig. 1). When the cells were treated with monensin

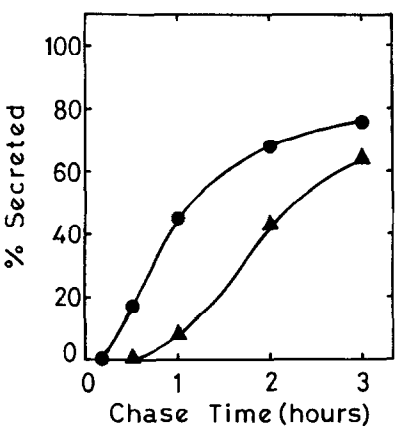

Fig. 1. Time course of haptoglobin secretion in cultured rat hepatocytes in the presence or absence of monensin. Hepatocytes were pulse-labeled for 10 min with [^{35}S]methionine and chased in the absence (●) or presence (▲) of monensin (1×10^{-6} M) as described in Methods. At the indicated times, immunoprecipitates of haptoglobin were prepared from cell lysates and medium, and determined for radioactivity. Values are expressed as percentages of the radioactivity secreted of the total radioactivity incorporated into haptoglobin during 10 min pulse (chase 0); 100% represents 6.1×10^4 dpm/dish for control cells and 5.7×10^4 dpm /dish for monensin-treated cells.

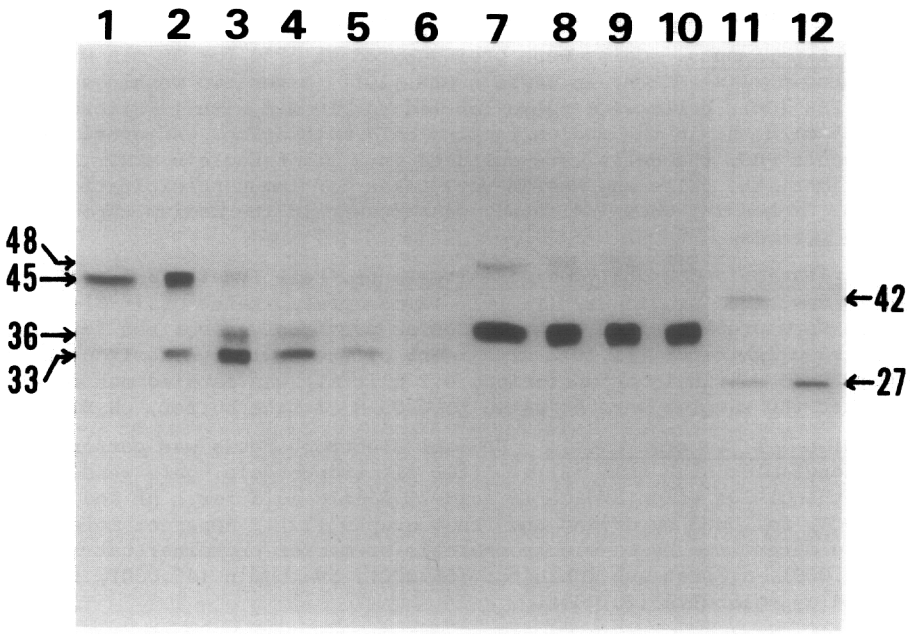

Fig. 2. SDS-gel electrophoresis of [^{35}S]-labeled haptoglobin synthesized by control cells. Immunoprecipitates of haptoglobin from the control cells were subjected to SDS-polyacrylamide gel (12.5%) electrophoresis, followed by fluorography. Lane 1, cellular haptoglobin from the cells pulse-labeled for 5 min with no chase. All the other samples were from the cultures with a 10 min pulse. Lanes 2-6, cellular haptoglobin obtained at 0, 30, 60, 120, and 180 min of chase, respectively; lanes 7-10, medium haptoglobin obtained at 30, 60, 120, and 180 min, respectively. Lanes 11 and 12, cellular (0 chase) and medium (60 min) haptoglobin, respectively, prepared from the cells treated with tunicamycin (5 μg/ml). Apparent molecular weights (x 10^{-3}) are shown on both sides of gel.

(1×10^{-6} M), the secretion was greatly inhibited up to 1 hr, after that the protein was rapidly secreted and reached about 86% of the normally secreted level compared at 3 hr of chase (Fig. 1). Such time-dependent escape from the secretion blockade by monensin seems to be a phenomenon observed depending on the cell types used; there have been two cases in rat hepatocytes (23) and human fibroblasts (24) so far reported.

Immunoprecipitates of intracellular and medium haptoglobin prepared from the control cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Fig. 2). Haptoglobin was initially synthesized as a major form having Mr=45Kd, although a minor band with Mr=33Kd also appeared (lanes 1-2). Relative intensity of the two bands varied depending upon the pulse times; a shorter pulse (5 min, lane 1) produced a relatively intense band of the 45Kd

form compared with the other (10 min, lane 2). When the cells were chased, already at 30 min the 45Kd form remained only as a trace level, concomitantly the 33Kd form became a major band (lane 3). In addition, there appeared another form with Mr=36Kd (lanes 3-6), which was secreted into the medium as a major form (lanes 7-10) and found to have the identical mobility with the β-subunit of the serum haptoglobin. In tunicamycin-treated cells, haptoglobin was also initially synthesized as two forms, 42Kd and 27Kd (lane 11), and the smaller one was secreted into the medium with no change in its molecular size (lane 12). A preliminary experiment showed that Endo H treatment of the samples shown in lanes 1-2 converted the two forms with 45Kd and 33Kd into smaller forms with 42Kd and 27Kd, respectively, while the 36Kd form in the cell as well as in the medium was resistant to Endo H (data not shown). Taken together, these results indicate that rat haptoglobin was initially synthesized as a pro-form (45Kd) with high mannose oligosaccharide chains (25) which was immediately cleaved into two subunits, the β-subunit with 33Kd and the α-subunit, the latter of which was not identified here since no [^{35}S]methionine was incorporated into it (8,10). The 33Kd β-subunit was further processed to the 36Kd form with complex type oligosaccharide chains before it was secreted into the medium. Of particular interest is the finding that the pro-form with 45Kd was not completely cleaved into the subunits during intracellular transport(lanes 3-6) and secreted into the medium after it had also been fully glycosylated to the Endo H resistant form with 48Kd (lanes 7-10).

The carboxylic ionophore monensin has been demonstrated to block the secretory process at the Golgi complex (13). We previously reported that the drug inhibits not only secretion but also proteolytic processing of proalbumin (11) as well as complete glycosylation of α_1-protease inhibitor (25), resulting in delayed secretion of their premature forms (23). The similar blockade by monensin has been observed in the proteolytic conversion from pro-opiomelanocortin to adrenocorticotropic hormone and β-endorphin (12), and from the p62 protein of Simliki Forest virus to E2 and E3 proteins (26). For comparison with these results, we examined the monensin effect on the haptoglobin processing.

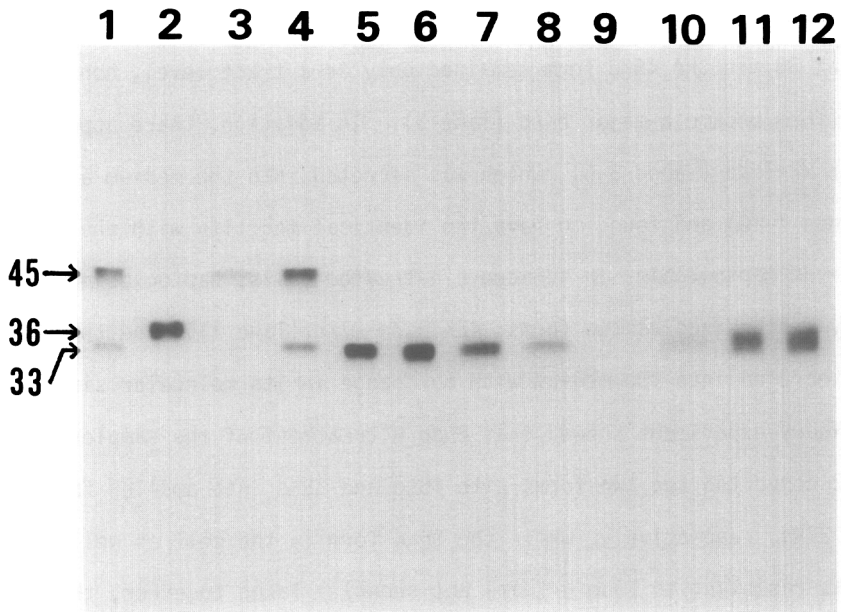

Fig. 3. Effect of monensin on intracellular processing of haptoglobin. Immunoprecipitates from the monensin-treated cells were analyzed by SDS-gel electrophoresis. Lane 3, cellular haptoglobin from the cells pulse-labeled for 5 min with no chase. All the other samples were from the cultures with a 10 min pulse. Lanes 4-8, cellular haptoglobin obtained at 0, 30, 60, 120, and 180 min of chase, respectively; lanes 9-12, medium haptoglobin at 30, 60, 120, and 180 min, respectively. Lanes 1 and 2, the same samples as those in lanes 2 and 10, respectively, in Fig. 2.

As shown in Fig. 3, the conversion of the 45Kd form to the 33Kd form took place in the monensin-treated cells essentially in the same manner as in the control cells (Fig. 2). Minor bands, intracellular 45Kd and medium 48Kd, remaining in chase of the control culture, were no longer detected in the monensin-treated cells. The most remarkable difference, however, was that there was no appearance of the 36Kd form in the treated cells as well as in the medium, where the 33Kd form was secreted. Thus, the results demonstrate that monensin never inhibited the porteolytic processing of the haptoglobin pro-form but rather accelerated it, although the drug blocked the glycosylic processing of the protein from high mannose type (33Kd) to complex type (36Kd) as observed for other proteins (23,25-27). A rapid conversion of the pro-form to the sub-units after pulse labeling (Fig. 2) and no blocking effect by monensin (Fig.3) consistently support the conclusion that its proteolytic processing is a very

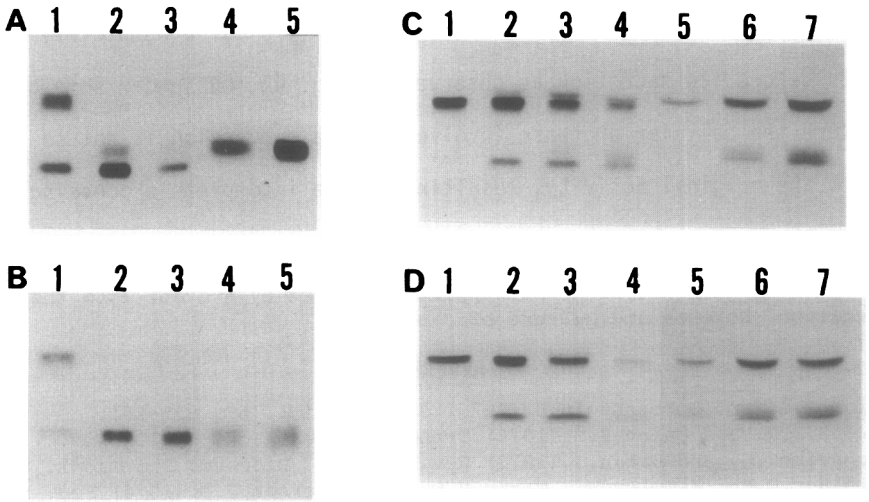

Fig. 4. Two extreme cases of proteolytic processing of haptoglobin. Two separate preparations of hepatocytes (A-B and C-D) were cultured, pulse-labeled, and chased in the absence (A and C) or presence (B and D) of monensin, followed by immunoprecipitation of haptoglobin and SDS-gel electrophoresis/fluorography, as described in Methods. In A and B; lanes 1-3, cellular haptoglobin taken at 0, 30, and 60 min of chase, respectively; lanes 4 and 5, medium haptoglobin at 30 and 60 min, respectively. In C and D; lanes 1-4, cellular haptoglobin taken at 0, 30, 60, and 120 min of chase, respectively; lanes 5-7, medium haptoglocin at 60, 120, 180 min, respectively.

early post-translational event, possibly within endoplasmic reticulum, different from other cases mentioned above occurring in the Golgi complex (4-6,11,12,26).

As shown in Fig. 2, the pro-form with 45Kd was not completely cleaved into the subunits, and its fully glycosylated form (48Kd) was secreted into the medium. A calculation from the densitometric profiles showed that the amount of the pro-form secreted was usually about 8% of the total secreted haptoglobin at 30 min of chase (lane 7). Although these results were quite reproducible under the conditions employed here, we have experienced two extreme cases of the proteolytic processing in performance of 10 separate experiments in which the cultured cells showed a similar capacity of protein synthesis and secretion. One is a case in which all the pro-form was completely converted to the subunits by 30 min of chase (Fig. 4A and B). The other is an opposite case in which most of the pro-form remained uncleaved and was secreted into the medium after its conversion to the 48Kd (Fig. 4C). The incompleteness of conversion could not be so effectively compensated by monensin treatment (Fig. 4D). Although the precise reason

for such discrepancy is unclear at present, these results suggest the possibility that in the liver tissue the pro-form is primarily cleaved completely into the subunits before its secretion as observed in Fig. 4A, but under some conditions in culture the cellular protease involved in the conversion is not so stable to exert the original activity, resulting in the incomplete processing of the pro-form to various extents.

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