Proteolytic Cleavage of Haptoglobin Occurs in a Subcompartment of the Endoplasmic Reticulum: Evidence from Membrane Fusion In Vitro

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Abstract. The primary translation product of haptoglobin mRNA is a 45-kD polypeptide which is proteolytically cleaved shortly after its synthesis. Previous studies have indicated that the cleavage of this proform of haptoglobin occurs in the ER. In an attempt to characterize the cleaving enzyme, we found that upon incubation of microsomes from rat hepatocytes pulse labeled with [^{35}S]methionine, little cleavage of labeled prohaptoglobin occurred. In contrast, when cells whose cytoplasmic proteins had been released by saponin treatment were incubated, 30–40% of the prohaptoglobin was cleaved. The addition of GTP caused a twofold stimulation, which was abolished by the nonhydrolyzable analog GTPγS. With a homogenate of the cells, the addition of GTP resulted in a fourfold stimulation of the degree of cleavage—from 15 to 60%. Differential centrifugation revealed that most of the cleaving activity resided in membranes sedimenting similarly to mitochondria and to a small fraction of the ER. These rapidly sedimenting membranes were therefore prepared from a rat liver homogenate. Upon treatment with high salt, light membranes were released which, when incubated with microsomes of pulse-labeled hepatocytes in the presence of detergent (and in the absence of GTP), induced specific cleavage of prohaptoglobin. The cleaving enzyme had an alkaline pH optimum indicating that it was not of lysosomal origin. These results suggest that cleavage of prohaptoglobin occurs in a subcompartment of the ER. Apparently, the connection between this compartment and the bulk of the ER is broken upon saponin treatment or homogenization but can be reestablished through a process requiring GTP hydrolysis.

HAPTOGLOBIN occurs in serum as a tetramer consisting of two 38- and two 9-kD polypeptides, which are linked by disulfide bonds (11); in human serum two additional, larger forms exist (32). Haptoglobin is a homolog of the chymotrypsinogen family of serine proteases (15, 22) but differs from these proteins in that it appears to lack proteolytic activity (15). The function of haptoglobin in the blood is apparently to bind the free hemoglobin that is released upon intravascular lysis of red blood cells. The complex of these two proteins is removed from the blood stream by the liver (31). Haptoglobin is synthesized in hepatocytes as a 45-kD polypeptide which rapidly forms dimers (10). Shortly afterwards ($t_{1/2} \sim 10$ min) each polypeptide is proteolytically cleaved yielding the final tetrameric structure. The cleavage site is situated between an arginine and isoleucine residue in the sequence -Pro-Val-Gln-Arg-Ile-Leu-Gly-Gly- (8, 40, 46). The function of the haptoglobin cleavage is unknown; it is not necessary for transport because prohaptoglobin that escapes cleavage (7, 9) is transported at the same rate as cleaved haptoglobin (20). Earlier studies have indicated that the cleavage of haptoglobin takes place in the ER (10, 24, 27). This makes haptoglobin unique among secretory proteins, which are otherwise proteolytically cleaved in the Golgi compartment (GC).[1] Very little is known about the enzyme mediating the cleavage of prohaptoglobin (9). In this report we present evidence suggesting that this protein resides in a subcompartment of the ER.

Materials and Methods

Chemicals

[^{35}S]methionine and [^{14}C]methylated molecular mass standards were from Amersham International (Buckinghamshire, England), and nucleotides and their analogs from Boehringer Mannheim (Mannheim, Germany). Saponin (white pure) was from E. Merck (Darmstadt, Germany), and dextran T10 from Pharmacia, Uppsala, Sweden. Creatine phosphate and creatine phosphokinase were from Sigma Chemical Co. (St. Louis, MO). Antibodies against human haptoglobin were from Dakopatts (Glostrup, Denmark). Protein disulfide isomerase was isolated from rat liver essentially as described (16) and antibodies were raised in a rabbit.

1. *Abbreviations used in this paper*: GC, Golgi compartment; PMS, postmitochondrial supernatant.

Cell Culture

Rat hepatocytes were isolated and cultured in 35- or 60-mm petri dishes for 20–24 h as described (36).

Preparation of Cytosol

A rabbit liver was homogenized at 200 g/l in 250 mM sucrose, 20 mM Tris-HCl, pH 7.5, and was then centrifuged at 600 *g* for 10 min. The resultant supernatant was centrifuged at 14,500 *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 100,000 *g* for 2 h. This supernatant, which will be referred to as cytosol, was frozen in liquid N_2 and stored at −70°C. When indicated, the cytosol was dialyzed over night against 1 liter of 25 mM Hepes-NaOH, pH 7.4, 100 mM KCl with one change of buffer.

Labeling with [^{35}S] Methionine

Cells in 35- and 60-mm dishes were rinsed twice in Buffer 3 (34) and then 0.9 or 2.7 ml, respectively, of MEM (21) lacking methionine and $NaHCO_3$, and buffered with Hepes-NaOH, pH 7.4, was added. The dishes were then rocked for 20 min and 40–120 μCi of [^{35}S]methionine was added. After a further 10-min incubation, the dishes were placed on ice.

Cleavage of Prohaptoglobin in Permeabilized Cells

Pulse-labeled hepatocytes were rinsed twice with cold PBS and were then incubated for 30 min at +6°C with 1.0 ml PBS containing 100 μg/ml saponin and 150 mg/ml dextran T10. The cells were then rinsed twice with a "cytosolic" buffer containing 25 mM Hepes-NaOH, 38 mM K-glucomate, 38 mM K-glutamate, 38 mM K-aspartate, 2.5 mM $MgCl_2$, 0.6 mM EGTA, 0.38 mM $CaCl_2$, 100 mg/ml dextran T10 (final pH of 7.4). 1 ml of the same buffer, containing other components when indicated, was then added and the dishes were rocked at 37°C for different time periods. When the effect of ATP was studied, an ATP regenerating system containing 30 μM ATP, 32 mM creatine phosphate, and 20 IU/ml creatine phosphokinase was used. When cytosol was included, its final protein concentration was 1.8 mg/ml. After the incubation the dishes were put on ice, the medium was removed and the cells were rinsed twice with cytosolic buffer. Finally, the cells were solubilized and immunoprecipitation, SDS-PAGE and fluorography were done as described previously (27).

Cleavage of Prohaptoglobin in Homogenate

Cells in two 60-mm dishes were pulse-labeled, rinsed twice with 250 mM sucrose and once with 50 mM sucrose. Then 400 μl of 50 mM sucrose was added to the cells which were scraped off and homogenized with 15 up-and-down strokes of a Dounce glass homogenizer (Kontes Glass Co., Vineland, NJ) with a tight-fitting pestle. Sucrose (65% by mass) was added to give a final concentration of 8% and the homogenate was mixed by another five strokes. Then 50 μl of the homogenate was mixed with 50 μl of a twofold-concentrated cytosolic buffer (described above) and 10 μl of 2.5 mM GTP. The mixture was then incubated for various times at 37°C. Finally, 10 μl of a solution containing 200 g/liter Triton X-100 and protease inhibitors was added and immunoprecipitation, SDS/PAGE and fluorography were done as described previously (27).

Differential Centrifugation

Aliquots (200 μl) of the homogenate from labeled cells were centrifuged at 1,000 or 1,700 *g* for different time periods. The supernatants were withdrawn and assayed for prohaptoglobin cleavage (as described above) and for various organelle markers (see below). For the measurement of the cleavage activity in pelleted membranes, a homogenate of labeled and unlabeled cells were centrifuged for 20 min at 1,000 *g*. Then 50 μl of the supernatant was mixed with a pellet obtained from 200 μl of homogenate from unlabeled cells.

Protein and Enzyme Assays

Protein concentrations were determined with a dye binding assay as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard. Succinate dehydrogenase, a marker for mitochondrial outer membranes, was assayed essentially as described (12). Samples of the supernatants from differential centrifugation (10 μl) were mixed with 600 μl of a solution containing 55 mM Na-phosphate, 1 mM KCN, 1 g/liter cytochrome c, 15 mM Na-succinate, pH 7.4. The decrease in absorbance at 552 nm was then measured. Nuclei were stained with Trypan blue and counted in a Bürker chamber.

Immunoblotting

Aliquots of supernatants from the differential centrifugation experiments were mixed with the same volume of a twofold-concentrated sample buffer for SDS-PAGE (36) and heated for 3 min at 95°C. After cooling, 5 μl of 0.2 M iodoacetic acid was added and the samples were run on a 10% polyacrylamide gel. Electrophoretic transfer and immunostaining were done as describe earlier (36) with antibodies against protein disulphide isomerase. The bands were analyzed with an Ultrascan XL densitometer (LKB-Pharmacia). Quantitation was done within the linear range of the assay.

Cleavage in Free Solution

ER membranes in the mitochondrial fraction were prepared as described by Shore and Tata (35). Briefly, the postnuclear supernatant from a rat liver homogenate was centrifuged at 6,000 *g* for 10 min. The pellet was resuspended in buffer containing 0.5 M KCl and 20 mM EDTA, homogenized with an Ultraturrax and centrifuged at 6,000 *g* for 10 min. The membranes in the supernatant were pelleted by a 60-min centrifugation at 100,000 *g* and were then treated for 15 min on ice with a solution containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA and 2 g/liter Triton X-100 or 20 g/liter saponin. Finally the sample was centrifuged for 60 min at 100,000 *g* and the supernatant collected. Different amounts of the detergent extract were then mixed with 25 μl extract of microsomes from labeled cells (see below) in a total volume of 75 μl. The samples were incubated for 16 h at room temperature and cleavage of prohaptoglobin was measured as described above. Microsomes from the postmitochondrial supernatant of rat liver and pulse-labeled hepatocytes were pelleted by a 60-min centrifugation at 100,000 *g* and extract as described above.

Density Gradient Centrifugation

ER membranes isolated from the mitochondrial fraction were suspended in 2.0 ml of 1.8 M sucrose and layered between 250 μl of 2.25 M-sucrose and 13 ml of a linear gradient of 1.0–1.55 M-sucrose in a centrifuge tube. After 16 h centrifugation at 100,000 *g* 1.5-ml fractions were eluted from the bottom. Each fraction was diluted to 5 ml with PBS and centrifuged for 60 min at 100,000 *g*. The pellets were solubilized in 100 μl of a buffer containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA and 2 g/liter Triton X-100. The content of protein disulfide isomerase and haptoglobin cleavage activity were measured as described above.

pH Dependence

Microsomes from pulse-labeled cells were solubilized with detergent and mixed with a saponin extract of the mitochondria-associated ER membranes in the presence of different buffers—Na-acetate, pH 4–6, NaP_i, pH 6–7, and Tris-HCl, pH 7–9—also containing 1.0 mM EDTA and 2 g/liter Triton X-100. The samples were then assayed for cleaving activity as described above. The final pH was determined in 1.0 ml samples prepared in parallel which lacked the membrane material.

Results

Cleavage of Prohaptoglobin in Permeabilized Cells

In an initial attempt to characterize the enzyme that cleaves prohaptoglobin, we isolated microsomes of pulse labeled hepatocytes, incubated them in the presence of detergent and then analyzed for cleavage by immunoprecipitation and SDS-PAGE followed by fluorography. This procedure yielded little cleavage of prohaptoglobin which we interpreted as being due to the great dilution of the enzyme and its substrate that occurred upon solubilization. However, incubation of intact microsomes also resulted in limited cleavage, which suggested that the cleaving enzyme resided in a compartment separate from that in which synthesis took place. We therefore decided to study the reaction in permeabilized cells. For

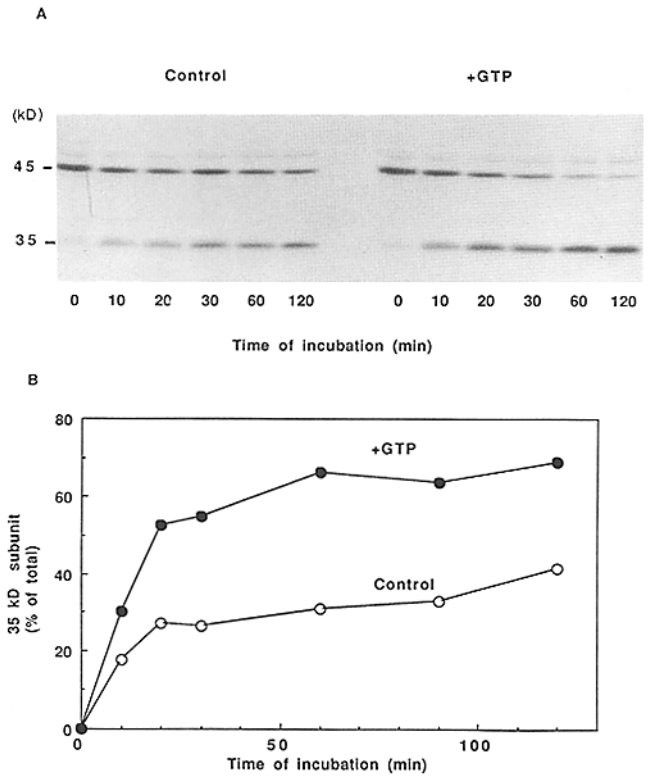

Figure 1. Prohaptoglobin cleavage in permeabilized cells. Isolated rat hepatocytes were pulse-labeled with [^{35}S]methionine. Their plasma membranes were then made permeable to proteins with saponin and the cells were incubated for different times in buffer alone (*control*) or in buffer supplemented with 200 μM GTP. Finally, the cells were solubilized and haptoglobin was immunoprecipitated and detected by SDS-PAGE followed by fluorography (*A*). The molecular masses of prohaptoglobin and the larger cleavage product are shown to the left. B shows the average, relative amounts of cleaved prohaptoglobin (35-kD band) obtained by densitometric scanning of the fluorographs from two experiments; the amount of cleaved haptoglobin in cells not incubated (10–20%) was subtracted from all values.

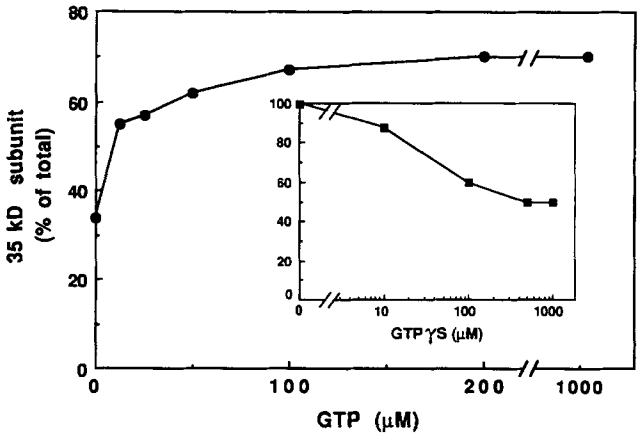

Figure 2. Concentration dependence of GTP on cleavage of prohaptoglobin in permeabilized cells. Pulse-labeled hepatocytes were treated with saponin and incubated for 60 min at 37°C at different concentrations of GTP. The extent of cleavage was then determined as described as in Fig. 1. (*Inset*) Effect of nonhydrolyzable GTP analog (*GTPγS*) on cleavage. Pulse-labeled hepatocytes were permeabilized and incubated for 60 min in the presence of 200 μM GTP and different concentrations of GTPγ*S*. The relative amount of the 35-kD subunit was then determined as described in Fig. 1. Cleavage in the absence of the analog was set as 100%.

this purpose pulse-labeled rat hepatocytes were treated at 6°C with saponin under conditions such that all soluble cytoplasmic material was released but no leakage of secretory proteins from the ER occurred (41); dextran was included in the incubation medium to preserve the flattened structure of the ER (42). The cells were then incubated at 37°C for various times and assayed for prohaptoglobin cleavage. Fig. 1 *A* shows that incubation in buffer only (*control*) resulted in significant cleavage of the 45-kD prohaptoglobin polypeptide. (The 9-kD polypeptide that is also formed upon cleavage does not contain [^{35}S]methionine and was therefore not visualized.) In an attempt to improve the efficiency of the reaction, we added GTP to the incubation mixture. As shown by Fig. 1 *A* this nucleotide clearly stimulated the cleavage. Quantitative data obtained by densitometric scanning of the fluorographs showed that 30–40% of prohaptoglobin was cleaved after 120 min of incubation in buffer only and that the extent of cleavage was twofold higher in the presence of GTP (Fig. 1 *B*). Half maximal cleavage was obtained after the first 10–20 min of incubation. Half maximal stimulation by GTP upon 60 min of incubation was achieved at a concentration of 10–25 μM (Fig. 2).

Cleavage Is Dependent on GTP Hydrolysis

When the permeabilized cells were incubated with the nonhydrolyzable analog GTPγS, no stimulation of the cleavage was obtained (not shown), indicating that it required hydrolysis of GTP and not just binding of the nucleotide. Furthermore, when GTPγS was added together with GTP, the reaction was inhibited. To quantify the inhibitory effect of GTPγS, we incubated the cells in the presence of 200 μM GTP and different concentrations of the analog. Fig. 2, *inset* shows that at the two highest concentrations of GTPγS used, the degree of cleavage was half its maximal value, demonstrating that GTPγS fully inhibits the GTP-dependent part of the cleavage reaction; half-maximal inhibition was obtained at 10–100 μM of GTPγS.

ATP and Cytosol Dependence

The possible ATP dependence of the cleavage reaction was also studied (Fig. 3). ATP alone did not have any effect (Fig. 3, bar *3*), whereas in the presence of cytosol it stimulated the reaction as much as GTP did (compare bars *4* and *2*). If dialyzed cytosol was used, however, the stimulation was considerably lower (Fig. 3, bar *5*). A simple explanation of these observations is that ATP itself is not required, but that in the presence of cytosol, an ATP-requiring conversion of endogenous GMP and GDP to GTP will take place, leading to stimulation of the cleaving reaction. In support of this idea, the addition of GTPγS abolished the stimulation obtained with cytosol and ATP (Fig. 3, bar *6*). Furthermore, we found that cytosol was inhibitory to the cleavage when mixed with GTP (Fig. 3, bar *7*), and that this effect was diminished by dialysis (bar *8*). This inhibition could be caused by GMP and GDP present in the cytosol. It should be noted here that GTP, in

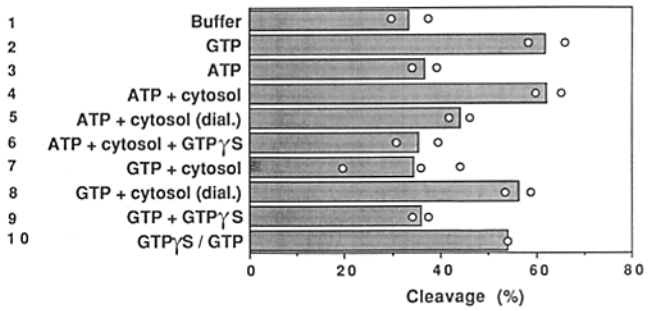

Figure 3. Characterization of cleavage of prohaptoglobin in permeabilized cells. Pulse-labeled hepatocytes were permeabilized and incubated in buffer containing no additives (*1*), 200 μM GTP (*2*), ATP (*3*), ATP and cytosol (*4*), ATP and dialyzed cytosol (*5*), ATP with cytosol and 400 μM GTPγS (*6*), 200 μM GTP and cytosol (*7*), 200 μM GTP with dialyzed cytosol (*8*), and 200 μM GTP and 400 μM GTPγS (*9*). When ATP was included in the assay, the ATP regenerating system described in Materials and Methods was used. The cells were also incubated with 400 μM GTPγS for 60 min, rinsed and incubated in 200 μM of GTP for another 60 min (*10*). The bars show average values of cleaved prohaptoglobin and the open circles indicate the results from each experiment.

contrast to ATP, was not maintained at a constant concentration with a regenerating system during the incubation. Part of the inhibitory effect of the cytosol when mixed with GTP could therefore be due to dephosphorylation by enzymes present in the cytosol. As already described in Fig. 2, GTPγS inhibited the GTP-dependent stimulation of the cleavage reaction (Fig. 3, bar *9*). To measure the reversibility of this inhibition, we first incubated the cells with GTPγS, then rinsed them and finally incubated with GTP alone (Fig. 3, bar *10*). The resultant cleavage was 80–90% of the maximal value, indicating that the block exerted by GTPγS could be partially reversed.

Cleavage Occurs in Rapidly Sedimenting Membranes

Having established conditions that yielded efficient cleavage

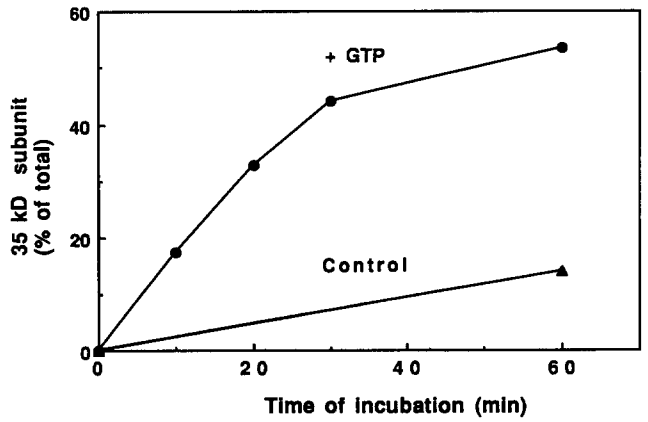

Figure 4. Time course of cleavage of prohaptoglobin in cell homogenate. Pulse-labeled hepatocytes were homogenized and incubated for up to 60 min in buffer alone (*control*) or in buffer also containing 230 μM GTP. Samples were taken after different times and the degree of cleavage was determined.

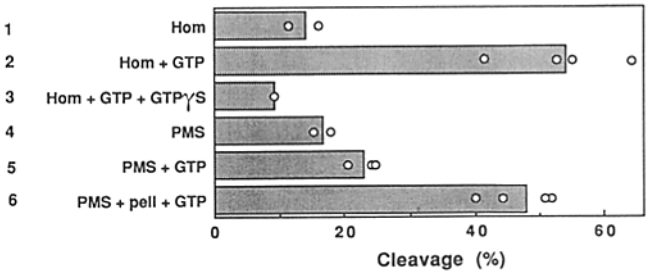

Figure 5. Characterization of cleavage in homogenate and postmitochondrial supernatant (*PMS*). Pulse-labeled hepatocytes were homogenized and aliquots were incubated for 60 min in buffer containing either no additives (*1*), 230 μM GTP (*2*), or 230 μM GTP and 300 μM GTPγS (*3*). One aliquot was centrifuged for 10 min at 1,700 *g*. The supernatant (PMS) was then mixed with buffer containing no additive (*4*) or 230 μM GTP (*5*). The pellet from a homogenate of unlabeled cells was resuspended in the PMS from labeled cells and incubated for 60 min in the presence of GTP (*6*). The bars show average values of cleavage and the open circles indicate the results from each experiment.

of haptoglobin in permeabilized cells, we tested whether the same conditions would also stimulate the reaction in a cell homogenate. Fig. 4 shows that in the presence of GTP, the cleavage in a homogenate was almost as efficient as in permeabilized cells (compare Figs. 1 and 4). In the absence of GTP, however, the degree of cleavage was only half of that observed for the permeabilized cells. GTPγS inhibited the reaction in the homogenate efficiently (Fig. 5, compare bars *3* and *2*) indicating that the nature of the reaction in the homogenate was the same as in the permeabilized cells. Unexpectedly, there was little activity in the postmitochondrial supernatant (*PMS*) obtained after a short centrifugation of the cell homogenate (Fig. 5, compare bars *5* and *2*). If a pellet, obtained by centrifugation of a homogenate from unlabeled cells, was resuspended in the PMS of the labeled cells, part of the activity was regained (Fig. 5, bar *6*).

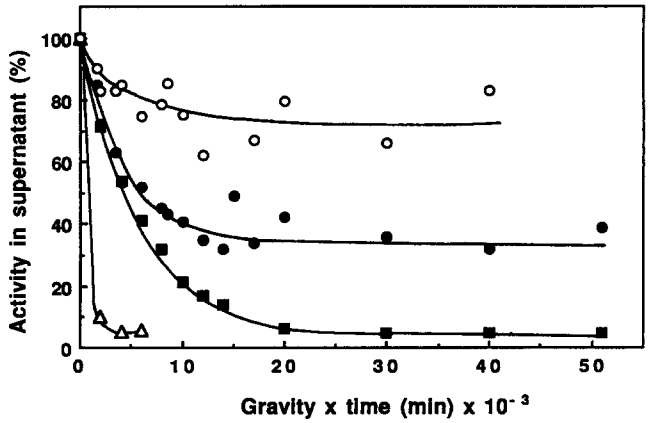

Figure 6. Effect of centrifugation of cell homogenate on cleavage activity. Pulse-labeled hepatocytes were homogenized and aliquots (200 μl) of the homogenate were centrifuged for different time periods. The supernatants were assayed for markers for the ER (protein disulfide isomerase, ○), mitochondria (succinate dehydrogenase, ■), nuclei (counted numbers, △) and prohaptoglobin cleaving activity (●). All variables are shown relative to the value obtained for the initial cell homogenate.

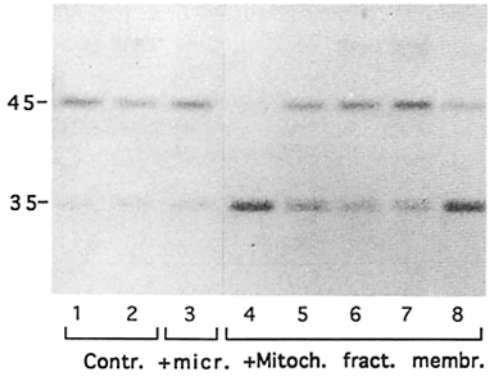

Figure 7. Cleavage in free solution. Microsomes from pulse-labeled hepatocytes were mixed with rat liver microsomes or with membranes released by high salt from the mitochondrial fraction of a rat liver homogenate. The mixtures were then incubated for 16 h in the presence of detergent and cleavage of haptoglobin was assessed as shown in Fig. 1. *A*. Lanes *1* and *2* show microsomes of pulse-labeled cells (20 μg) before and after incubation, respectively. Lane *3* shows the effect of adding rat liver microsomes (360 μg) to the incubation mixture. In lanes *4–7* decreasing amounts of membranes released from the mitochondrial fraction were added—480, 43, 8.6, and 4.3 μg of protein, respectively. In lane *8*, the effect of a saponin extract from the same amount of membranes as in lane *5* is shown.

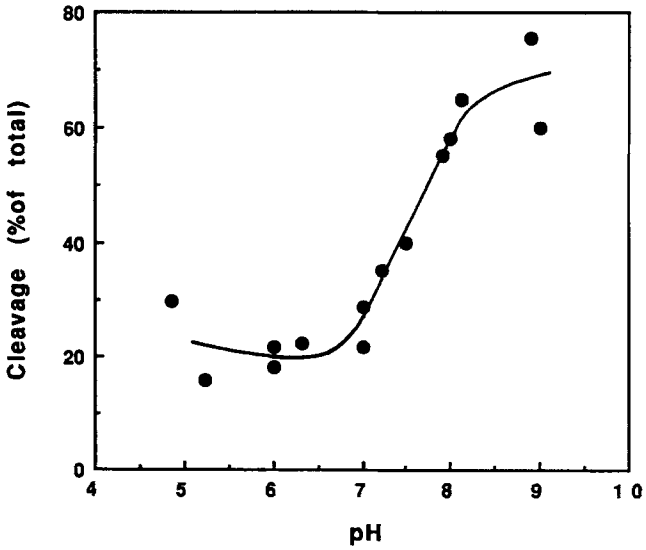

Figure 8. Effect of pH on cleavage in free solution. Microsomes from pulse-labeled hepatocytes were solubilized with detergent, mixed with a saponin extract of the membranes released from the mitochondrial fraction and buffers of different pH. After a 16-h incubation at different pH cleavage of haptoglobin was determined as described in Fig. 1. The results from two experiments are shown.

The sedimentation properties of the membranes containing the cleaving activity in a homogenate of labeled cells were then assessed by differential centrifugation (Fig. 6). For comparison, the supernatant was also assayed for cell nuclei, mitochondria, and ER membranes. Under conditions at which only 30–40% of the cleaving activity remained in the supernatant (20–40 × 10^3 *g* × min), 70–80% of the ER membranes were still in suspension; essentially the same result was obtained whether the marker was a soluble ER-specific protein (protein disulfide isomerase; as in Fig. 6), a membrane bound ER protein (NADPH-cyt c reductase), or pulse-labeled haptoglobin (not shown). The loss of cleaving activity from the supernatant upon centrifugation was not simply due to a time-dependent decrease in activity because it increased simultaneously in the pellet (not shown); however, the recovery of the activity was low (30–40%), suggesting that an essential factor was lost from the membranes upon suspension. Apparently, the major part of the cleaving activity as well as a minor fraction of the ER appeared to sediment with rates similar to that of mitochondria.

Cleavage in Free Solution

The ER membranes in a rat liver homogenate that are pelleted by a short centrifugation have been characterized by Tata and co-workers (18, 35). Their results suggest that at least part of these membranes are firmly attached to mitochondria and can be released only under harsh conditions. We used their procedure to isolate these ER membranes and then incubated them with microsomes from pulse-labeled cells and incubated the mixture in the presence of detergent. Fig. 7, lane *4* shows that upon prolonged incubation (16 h), the radiolabeled prohaptoglobin was efficiently converted into haptoglobin. When these membranes were replaced by a similar amount of microsomal membranes, however, cleavage was as low as in the control sample (Fig. 7, lanes *3* and *2*, respectively). With a decreasing amount of mitochondria-associated membranes, a decreasing degree of cleavage was obtained (Fig. 7, lanes *4–7*). Quantitative analysis of these results indicated that the specific activity of the mitochondria-associated membranes was at least 10-fold higher than that of microsomes. To test whether the cleaving enzyme was soluble or not, the membrane fraction was treated with saponin and the released active proteins were added to the incu-

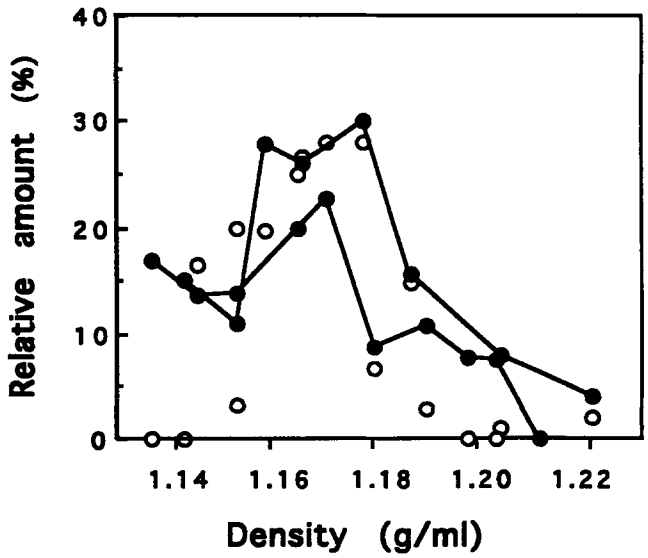

Figure 9. Equilibrium density centrifugation of membranes containing cleaving activity. Membranes released from the mitochondrial fraction were layered under a sucrose gradient and centrifuged at 100,000 *g* for 16 h. The membranes in the obtained fractions were then pelleted and solubilized. The relative amounts of protein disulfide isomerase (○) and prohaptoglobin cleavage activity (●) were determined. The results from two experiments are shown.

bation mixture. Fig. 7, lane *8* shows that prohaptoglobin was efficiently cleaved by this extract.

pH Dependence

The membrane fraction with a high cleaving activity also contained a small amount of lysosomal membranes, raising the possibility that the cleaving enzyme was derived from this organelle. One way of testing whether an enzyme is lysosomal or not is to determine whether it has an acidic pH optimum. Fig. 8 shows the pH dependence of the cleavage reaction in free solution. Clearly, the reaction rate increases markedly from pH 7 to 8. No further cleavage of either haptoglobin or albumin could be observed upon incubation at pH 8 (data not shown), demonstrating the specificity of the reaction. At pH 4, however, the prohaptoglobin was completely degraded (data not shown) indicating the occurrence of lysosomal proteases in the incubation mixture.

Buoyant Density of Membranes Containing Cleaving Enzyme

As a further characterization of the protease-containing membranes, we subjected them to equilibrium density centrifugation. The obtained fractions were assayed for the haptoglobin cleaving enzyme and protein disulfide isomerase, a marker for the ER. Fig. 9 shows that the distributions of the two proteins were essentially the same with maxima at 1.16–1.18 g/ml.

Discussion

The results presented in this study suggest that newly synthesized haptoglobin is cleaved in a subcompartment of the ER in hepatocytes. This conclusion is based on observations made with permeabilized cells and with cell homogenates. In the former case, soluble cytoplasmic components were released by saponin treatment under conditions that preserve the morphology of the ER (42). Upon subsequent incubation, partial cleavage of haptoglobin occurred and this reaction was stimulated twofold by the addition of GTP (Fig. 1). GTP has been shown to be required for the vesicular transport of proteins from the ER to the GC (2) but a number of observations indicate that the cleavage of newly synthesized prohaptoglobin is not preceded by transport to the GC. First, cleaved haptoglobin can be detected in the ER fraction of hepatocytes (10, 24, 27). Second, about half the pulse-labeled haptoglobin in intact cells is cleaved after 90 min of incubation at 16°C (our own unpublished observation), whereas transfer to the GC at this temperature is completely blocked (7). Third, cleavage is not affected by 10 mM azide, which blocks intracellular protein transport (27).

Studies on living cells have demonstrated that the ER is a highly dynamic structure (6, 14, 17). Thus, for example, it has the capacity to reversibly vesicularize during mitosis (47). Experiments with ER-derived vesicles have shown that GTP at physiological concentrations may induce membrane fusion (26) and that under appropriate conditions the vesicles will form a network (23). Based on these observations we suggest the following explanation for our findings with saponin-treated hepatocytes: when the cells are permeabilized, part of the connections between the bulk of the ER and the compartment containing the haptoglobin-cleaving enzyme are broken. Upon addition of GTP, these connections will be reestablished by membrane fusion. The part of haptoglobin that is cleaved upon incubation in the absence of GTP could be accounted for by the connections that are still intact. Indeed, when dextran, which preserves the membrane structure (42), was omitted from the permeabilizing buffer, the degree of cleavage in the absence of GTP was reduced (our own unpublished observation).

Using homogenized hepatocytes we found that in the presence of GTP, cleavage was as efficient as in permeabilized cells. Biochemical analysis of the transport of proteins within the GC in cell homogenates has shown that the initial step in the fusion of transport vesicles with an acceptor membrane is the removal of the protein from the vesicles (25). This process requires hydrolysis of GTP and is inhibited by GTPγS (19). In the subsequent step, soluble cytoplasmic proteins bind to the membranes and fusion ensues (33, 44, 45). It is possible that a similar mechanism operates in the system described in our work. The fact that the addition of cytosol did not provide any stimulation of the prohaptoglobin cleavage could be due to the necessary proteins already being bound to the participating membranes; the presence of GTP-binding proteins in hepatic microsomes has been demonstrated (5). Interestingly, Newport et al. (23), in studying the reformation of nuclear envelopes from extracts of mitotic cells, also found no requirement for soluble proteins. We have also found that preincubation of permeabilized hepatocytes with GTPγS together with cytosol irreversibly inhibits cleavage of prohaptoglobin (our own unpublished observation). Similarly, Boman et al. (3) have shown that incubation with GTPγS causes a cytosolic factor to irreversibly bind to vesicles derived from nuclear membranes and thereby inhibits their fusion. This factor could be one of the GTP-binding proteins belonging to the family of ADP ribosylating factors (ARF) (4) which have been suggested to play a role as regulators of fusion in vesicular transport (1, 28).

Differential centrifugation of homogenized hepatocytes showed that most of the cleaving activity resided in membranes sedimenting similarly to mitochondria. Furthermore, we found that ER-derived membranes could be prepared from the mitochondrial pellet of a homogenized rat liver and that these membranes contained a soluble enzyme that could specifically convert prohaptoglobin to haptoglobin in the presence of detergent. This enzyme did not seem to be of lysosomal origin since it had an alkaline pH optimum. Whether this is the enzyme that actually cleaves prohaptoglobin in vivo remains to be shown. At the moment we can only note that it does not require GTP for its activity, consistent with the idea that the nucleotide dependence observed for the reaction in permeabilized cells or in cell homogenate reflects a fusion process. The mean density of the membranes containing the haptoglobin cleaving enzyme was slightly lower (1.17 g/ml) than that of the bulk of the ER of rat liver (1.18–1.20 g/ml; 41), supporting the idea that they were derived from a subcompartment of this organelle. ER membranes that sediment like mitochondria in rat liver homogenates have previously been reported to contain specific enzymatic activities, albeit none associated with secretion (30, 39). One reason for their rapid sedimentation is that they appear to be directly attached to mitochondria (29, 30), another that they form stacks of flattened cisternae (35). While it is clear that newly synthesized haptoglobin enters the compartment containing the cleaving enzyme dur-

ing its intracellular transport, it remains to be shown whether this compartment represents an obligatory station of the secretory pathway. We have so far not been able to obtain any evidence in pulse–chase experiments that newly synthesized proteins pass through the rapidly sedimenting ER fraction on their way to the cell surface. However, these results are inconclusive since most of the membranes in this fraction may be derived from the bulk of the ER.

The protease that cleaves newly synthesized haptoglobin presumably has additional functions. Misfolded or otherwise aberrant proteins are prevented from leaving the ER and are eventually degraded (13, 38, 43). At least part of this degradation seems to take place in the ER (37) and it is conceivable that the haptoglobin-cleaving enzyme has a role in this process. Another possibility is that haptoglobin is cleaved by a secretory protease which becomes activated in a subcompartment of the ER (9). Clearly, further insight into the intracellular localization and function of this protein will require its isolation, a goal we are now pursuing.

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