Biosynthesis and Intracellular Transport of Rat Liver 5'-Nucleotidase*

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To investigate biosynthesis and intracellular transport of 5'-nucleotidase, we purified this enzyme from rat liver and prepared antibodies. In immunoblot analysis, 5'-nucleotidase from the plasmalemmal, Golgi, and tritosomal fractions migrated as a single band of 72 kDa. The immunoreactive 68-kDa band was detected in the rough microsomal fraction and only the 72-kDa polypeptide contained sialic acids. The single polypeptide of 61 kDa immunoprecipitated from the translation products with the membrane-bound polysomal RNAs by the cell-free system converted to the 68-kDa form associated with membranes, when translated in the presence of microsomal vesicles. 5'-Nucleotidase from cultured primary hepatocytes pulse-labeled with [35S]methionine for 20 min migrated as a 68-kDa polypeptide. Within 45 min of chase, the 68-kDa form was converted to the 72-kDa form. Upon treatment with tunicamycin, a new immunoreactive polypeptide of 59 kDa was obtained. These results suggest that 5'-nucleotidase is translated on the membrane-bound polysomes as a 61-kDa precursor and that the cleavage of the 2-kDa signal peptide and the core glycosylation co-translationally convert the precursor to the 68-kDa form, which is subsequently processed in the Golgi complex to the 72-kDa form.

5'-Nucleotidase (EC 3.1.3.5, 5'-ribonucleotide phosphohydrolase) is an integral plasma membrane glycoprotein (1-3) which hydrolyzes nucleotide 5'-monophosphate. It is also an ectoenzyme (4-7) with its active site facing the external medium rather than the cytoplasm. This enzyme has been widely used as a marker for plasma membrane (5). Sasaki et al. (8) found that adenosine is formed on the hepatocyte cell surface by the action of 5'-nucleotidase from extracellular nucleotides and is taken up into the intracellular compartment by the nucleotide transport protein, side by side with 5'-nucleotidase.

The plasma membrane has a highly differentiated structure and continues to exchange membranes with a pool of cytoplasmic membranes (9, 10) so that various cell surface phenomena can be expressed. Matsuura et al. (11) demonstrated that the surface density of 5'-nucleotidase is much higher on the bile canalicular surface than on the sinusoidal surface and is low on the lateral surface, and that 5'-nucleotidase is almost exclusively located on the microvilli, in large clusters, both on the bile canalicular and sinusoidal surface. They also suggested that 5'-nucleotidase on microvilli of the sinusoidal surface absorbs nucleotides from the blood plasma. Widnell et al. (12) and Stanley et al. (13) reported that 5'-nucleotidase circulates between the cell surface and an intracellular pool, independently of its synthesis and degradation, and that the exchange of 5'-nucleotidase between these pools was found to be inhibited in nonmammal-treated cells (14). While 5'-nucleotidase is a well known protein in the plasma membrane, its biosynthesis, processing, and routing to the cell surface are poorly understood.

We investigated the biosynthesis of 5'-nucleotidase, using an in vitro translation system and primary hepatocyte culture.

EXPERIMENTAL PROCEDURES

RESULTS

Immunoblotting of 5'-Nucleotidase—To characterize the size of 5'-nucleotidase in subcellular fractions, we used the technique of immunoblotting. 5'-Nucleotidase was mainly localized in the plasma membrane fraction and had a molecular weight of 72,000 (mature form) (Fig. 3, lane 1). After treatment of the plasma membranes with neuraminidase, the molecular weight became 68,000, thereby indicating that 5'-nucleotidase in the plasma membrane is a sialoglycoprotein (Fig. 3, lane 2). We examined the size of 5'-nucleotidase in the rough endoplasmic reticulum. Since the content of 5'-nucleotidase in this fraction was low, 5'-nucleotidase was isolated by anti-5'-nucleotidase IgG-Sepharose beads followed by immunoblotting. The fraction contained a polypeptide of 68 kDa which co-migrated with the asialo-form of 72 kDa (Fig. 3, lanes 3 and 4). Only the microsomal 68-kDa form could be converted to 60 kDa by digestion with endo-β-N-acetylgalosaminidase H which cleaves high mannose oligosaccharides leaving one N-acetylgalatosamine residue per oligosaccharide from N-linked glycoprotein (32) (data not shown). Since the amount of 5'-nucleotidase in the endoplasmic reticulum was about 100 times lower than that in the

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The abbreviations used are: Con A, concanavalin A; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

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plasma membranes, the mature form obtained in the microsomal fraction would be due to contamination of the latter. Therefore, the 5'-nucleotidase in the endoplasmic reticulum would be a 68-kDa polypeptide bearing high mannose oligosaccharides. In the Golgi fraction, only the mature form, which was sialylated, was obtained (Fig. 4, lanes 3, 4, and 5). Therefore, the enzyme was probably synthesized on the rough endoplasmic reticulum, glycosylated, moved to the Golgi complex where the carbohydrate portion of the enzyme was processed, and then transported to the plasma membrane.

In Vitro Synthesis of 5’-Nucleotidase.—To explore the processing of the enzyme in the endoplasmic reticulum, we carried out an experiment in which a cell-free protein-synthesizing system was used with rat liver mRNAs. Poly(A)-containing RNAs were translated in a rabbit reticulocyte lysate system containing [35S]methionine. Translation products (containing 5 × 10⁶ cpm) were immunoprecipitated with anti-5’-nucleotidase antibodies (lane 1), as described under “Experimental Procedures.” The immunoprecipitates were analyzed by SDS-gel electrophoresis followed by fluorography for 1 month. In lane 2, immunoprecipitation was carried out in the presence of large excesses of purified 5’-nucleotidase. The arrow points to the position of the mature form of 5’-nucleotidase.

We attempted to determine whether 5’-nucleotidase is synthesized on ribosomes associated with membrane or on free ribosomes. Membrane-bound or free polysomes were prepared in accordance with the procedure of Ramsey and Steele (20) and poly(A)-containing RNAs were purified from each polysome. The immunoprecipitated translation product from each mRNA was analyzed (Fig. 6, lanes 1 and 2). The 61-kDa polypeptide was detectable only in translations of the membrane-bound mRNA pool, thereby providing support for the proposal that the 61-kDa polypeptide was the precursor of 5’-nucleotidase.

Dog pancreas microsomal vesicles were added to the cell-free system after or during translation in order to examine integration into the microsomal membranes and glycosylation of the precursor. No change in the translation product was observed when the membranes were added post-translationally (Fig. 6, lane 3). When the membranes were added co-
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**Fig. 6.** *In vitro* translation of poly(A)-containing RNA from free and bound polysomes and the effect of the addition of microsomal vesicles. Free and membrane-bound polysomes were separated as described under "Experimental Procedures." Translation products (containing $5 \times 10^6$ cpm) were added after the completion of translation and the preparation was incubated at 25 °C for 60 min. mRNAs from membrane-bound polysomes were translated in the presence of dog pancreas microsomal vesicles (4 A$_{260}$/ml) (lane 4). After translation in the presence of membranes, the mixtures were brought to pH 11.5 by the addition of 0.1 N NaOH, incubated at 0 °C for 15 min, then separated into membrane and supernatant by a 150,000 × g centrifugation after neutralization with 1 N HCl (lanes 5 and 6, respectively). Markers on the left denote (top to bottom) 68, 55, and 43 kDa.

**Fig. 7.** Pulse-chase analysis of 5'-nucleotidase in cultured primary hepatocytes. Cultured primary hepatocytes were pulsed for 20 min with 100 μCi of [35S]methionine and chased with cold methionine for the indicated times (0–90 min). Thereafter, cells were solubilized, immunoprecipitated, and analyzed by SDS-gel electrophoresis followed by fluorography, as described under "Experimental Procedures."

**Fig. 8.** Effect of tunicamycin on the synthesis of 5'-nucleotidase in cultured primary hepatocytes and endo-β-N-acetylglucosaminidase H digestion. Cultured primary hepatocytes were preincubated for 16 h with 2 μg/ml tunicamycin, then labeled with [35S]methionine for 30 min in the presence of tunicamycin and solubilized, followed by immunoprecipitation (lane 3). In lanes 1 and 2, hepatocytes were labeled for 20 min, then solubilized and immunoprecipitated. The immunoprecipitates were treated with (lane 1) or without (lane 2) endo-β-N-acetylglucosaminidase H, as described under "Experimental Procedures." Samples were analyzed on SDS gel followed by fluorography. The marker points to the position of the primary translation products.

translationally, the 61-kDa band was converted to 68 kDa which corresponded to the microsomal form (Fig. 6, lane 4).

Since a polypeptide integrated into membranes is resistant to alkali extraction, under conditions where peripherally associated protein is solubilized (33, 34), this procedure was used to test membrane integration of the 68-kDa product. After completion of translation conducted in the presence of the microsomal vesicles, the vesicles isolated by centrifugation were brought to pH 11.5 by the addition of 0.1 N NaOH, incubated at 0 °C for 15 min, and then separated into membrane and supernatant by a 150,000 × g centrifugation after neutralization with 0.1 N HCl (lanes 5 and 6, respectively). The 68-kDa product was recovered on the membrane fraction. These results indicate that 5'-nucleotidase synthesized on the rough endoplasmic reticulum was integrated into the endoplasmic reticulum membrane and core-glycosylated co-translationally.

**Biosynthesis of 5'-Nucleotidase in Cultured Primary Hepatocytes**—Biosynthesis of 5'-nucleotidase was studied in pulse-chase experiments. The cultured hepatocytes were pulsed with [35S]methionine for 20 min and either directly lysed or chased with unlabeled methionine. After 20 min of labeling, only the 68-kDa polypeptide was recovered by immunoprecipitation. Following 15 min of chase, about half of the 68-kDa form was practically converted to the mature form and the 68-kDa form was almost totally converted to the mature form for up to 45 min of chase (Fig. 7). When the immunoprecipitated product at 20 min of pulsing was digested with endo-β-N-acetylglucosaminidase H, the 60-kDa polypeptide was recovered (Fig. 8, lane 2). These results show that the 61-kDa primary translation product was converted to the 68-kDa form by the
addition of high mannose oligosaccharide in the endoplasmic reticulum and the glycosylated protein was transported into the Golgi complex, where the oligosaccharide portion converted to the complex type. Fifteen to forty-five min was required for the 5'-nucleotidase in the endoplasmic reticulum to reach the trans side of the Golgi apparatus where the sialyltransferase locates.

To determine whether or not the signal peptide was cleaved off, we used tunicamycin and cultured primary hepatocytes. This drug blocks the dolichol-dependent glycosylation (35). Cells were preincubated with tunicamycin for 16 h and labeled for 30 min in the presence of this drug. This treatment of the cells with tunicamycin inhibited the formation of the 68-kDa form and yielded a new type of immunoreactive polypeptide of 59 kDa (Fig. 8, lane 3). We consider that about a 2-kDa signal polypeptide was cleaved off from the preform (61 kDa) and that the apparent molecular mass of the sugar moiety in the microsomal 5'-nucleotidase was 9 kDa.

**DISCUSSION**

The objective of our experiments was to elucidate events involved in the biosynthesis of 5'-nucleotidase in rat liver. We combined three types of experiments, immunoblot analysis, the cell-free protein-synthesizing system programmed with rat liver mRNAs, including the addition of dog pancreas microsomal vesicles, and pulse-chase experiments with primary hepatocyte cultures.

The presence of 5'-nucleotidase in both the endoplasmic reticulum and the Golgi apparatus has been demonstrated using cytchemical and biochemical procedures (36-38). It can be assumed that 5'-nucleotidase synthesized in the endoplasmic reticulum passes through the Golgi complex to the cell surface. The availability of monospecific antibodies against the purified 5'-nucleotidase facilitated study of the biosynthetic pathway.

Fig. 9 is a diagram of the processing and intracellular transport of 5'-nucleotidase derived from the results. Immunoblotting of the rough microsomal vesicles revealed a 72-kDa form as well as the 68-kDa form (Fig. 3, lane 3) which contained high mannose oligosaccharides and was susceptible to digestion with endo-β-N-acetylglucosaminidase H. We regard the mature form to be a contamination derived from plasma membranes. Since the specific activity of 5'-nucleotidase in the plasma membrane fraction is about 100 times higher than that in the rough microsomal fraction, an equal or larger amount of the mature form should have been visible in immunoblotting of the rough microsomal fraction when only 1% of the plasma membrane is contaminated in the rough microsomal fraction. The 72-kDa form was converted to the 68-kDa form by treatment with neuraminidase; therefore, this 68-kDa form must possess complex type oligosaccharides. We assumed that an immature form of 5'-nucleotidase would be present in the immunoblotting of the Golgi fractions, especially in the cis Golgi-rich fraction, GF3. However, only the mature form was detected in the three fractions (Fig. 4). These results suggest that the enzyme rapidly reaches the trans Golgi region where terminal glycosylation is known to occur. The detection of the 72-kDa form in the GF3 fraction may reflect contamination of the trans Golgi vesicles in this fraction.

Our results show that the biosynthetic pathway of 5'-nucleotidase would generally follow a route taken by other secretory or integral membrane proteins (39, 40). The primary translation product of 5'-nucleotidase had a slightly higher apparent molecular weight than did the unglycosylated polypeptide immunoprecipitated from cultured primary hepatocytes treated with tunicamycin, a drug which inhibits the N-linked core glycosylation. We regard the difference of the mobility, about 2 kDa, as the signal peptide cleaved from the translation product. This size is comparable with the reported length of the signal peptide, 15-30 amino acids (41). An integration of the primary translation product into membranes would lead to cleavage of the signal peptide and the core glycosylation, when the microsomal vesicles were added co-translationally (Fig. 6).

The difference between the mobility of the endo-β-N-acetylglucosaminidase H-treated microsomal form (68 kDa) and the product in the presence of tunicamycin (59 kDa) is shown in Fig. 8. Since this difference would be attributed to about 5 residues of N-acetylglucosamine remaining in the case of the former, we presumed that the 5'-nucleotidase contained about 5 asparagine-linked oligosaccharides. The same estimation for the number of oligosaccharides might be deduced from the data that the apparent molecular weight of the sugar moiety in the microsomal 5'-nucleotidase was 8 kDa (Fig. 8, lanes 1 and 3), although it is known that the migration through the gel tends to be anomalous by accompanying glycosylation of the protein.

Pulse-chase experiments showed that about half of the 5'-nucleotidase was sialylated after a 15-min chase following a 20-min pulsing and 5'-nucleotidase was almost totally sialylated after 45 min of chase. This indicates that the enzyme reaches the trans side of the Golgi complex where terminal glycosyltransferase locates. The time required for maturation is similar to that of vesicular stomatitis virus G protein (39), the erythrocyte anion transport protein (42), HLA-A and -B antigens (43), and low density lipoprotein receptor (44), and the enzyme moves to the plasma membranes faster than that in the case of asialoglycoprotein receptor (45), mannose 6-phosphate receptor (46), and HLA-DR antigen (47).

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**REFERENCES**

Cell-free Protein Synthesis — Poly(A)-containing RNAs were translated in a rabbit reticulocyte lysate system for 45 min at 30 °C. As described by Gonzales and Kasper (121), total counts incorporated into proteins were determined as described by Moore et al. (121). Dog pancreas microsomes, 100 ml of translation mixtures at 4 °C were added to translation mixtures at a final concentration of 4,140/ml. Translation mixtures were brought to 15 mO/0.5% Triton X-100/1 M NaCl/10 mM Hepes (pH 7.5) with the addition of 0.5% Triton X-100/0.15 M NaCl/25 mM EDTA/10 mM Tris·HCl (pH 7.5) (buffer A), and 15 μg/ml of protease inhibitors, leupeptin, chymostatin, pepstatin A and antipain (buffer A), Immunoprecipitation was carried out as described below.

Purification of 5'-Nucleotidase from Rat Liver and Antibody Preparation — Antiserum against the purified 5'-nucleotidase was raised on rabbits by the method of the Mo et al. (139, 140). The purified enzyme had a specific activity of 100 units/mg of protein (one unit hydrolyses 1 μmol of 5'-nucleotidase at 37 °C). Antiserum against the purified 5'-nucleotidase was applied on the 5'-nucleotidase-Sepharose 4B column equilibrated with 0.5% Triton X-100/0.15 M NaCl/10 mM Hepes (pH 7.5) (buffer B), containing 10 μg/ml of protease inhibitors, leupeptin, chymostatin, pepstatin A and antipain (buffer A). Immunoprecipitation was carried out as described below.

Subcellular Fractionation — Kistar male rats weighing 150 to 250 g were used for subcellular fractionations. Plasma membranes were isolated by the procedure of Ray (151), rough microsomes by the method of Keilin et al. (161), Golgi fractions by the method of Schwartz et al. (117). Marker enzymes were isolated from the rat liver homogenate in the presence of buffer A and washed with buffer B at 0 °C. The purified preparation showed a single band of apparent molecular weight of 72,000 on SDS gel electrophoresis. The antibodies were directed against this preparation in rabbits.

The reproducibility of the antibodies raised in rabbits was confirmed by complete precipitation of the enzyme (Fig. 2a) and by immunodiffusion (Fig. 2b). The specificity of the antibodies was confirmed by complete precipitation of the enzyme (Fig. 2a) and by immunodiffusion (Fig. 2b). The specificity of the antibodies was confirmed by complete precipitation of the enzyme (Fig. 2a) and by immunodiffusion (Fig. 2b). The specificity of the antibodies was confirmed by complete precipitation of the enzyme (Fig. 2a) and by immunodiffusion (Fig. 2b).
Fig. 1. SDS gel electrophoresis of purified 5'-nucleotidase. Purified 5'-nucleotidase (30 μg) was analyzed by the procedure of Weber and Osborn (48). Lane 1, stained with Coomassie brilliant blue; lane 2, stained with periodic acid-Schiff reagent.

Fig. 2: Specificity of anti-5'-nucleotidase antibodies. (A) Precipitation of 5'-nucleotidase from rat liver with antisera directed against 5'-nucleotidase. To each 50 μl of purified enzyme preparation containing 0.1 μg of enzyme protein were added 50 μl portions of 0.15 M NaCl/10 mM Tris-HCl (pH 7.5) containing various amounts of antibody. The resulting mixtures were incubated for 60 min at 37 °C, then for 18 hours at 4 °C. The precipitates were obtained by centrifugation at 15,000 rpm for 15 min in the Hitachi model MR-150 microfuge. The activity remaining in the supernatant was assayed. Protein determinations on the precipitates were not performed due to the low amounts of enzyme used. (B) Immunoblotting of the purified 5'-nucleotidase and 10,000 x g pellet of rat liver homogenate. Purified 5'-nucleotidase was analyzed by SDS gel electrophoresis. A 10,000 x g pellet of rat liver homogenate solubilized with 2% sodium lauroyl sarcosinate/5 mM Tris-HCl (pH 7.5) was incubated with anti-5'-nucleotidase antibodies-Sepharose 4B. 5'-nucleotidase was released with 2% SDS/50 mM Tris-HCl (pH 6.8) and reduced with 2-mercaptoethanol, then separated on SDS gel. The gel was blotted onto a nitrocellulose sheet and stained, as described under EXPERIMENTAL PROCEDURES. Lane 1, purified 5'-nucleotidase; lane 2, rat liver homogenate; lane 3, nonimmune IgG-Sepharose 4B was used in lane 2. 1 μg, heavy-chain of released IgG from antibody-Sepharose 4B.

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Immunoprecipitation - Translation mixtures or cell extracts in buffer A were preincubated with 100 μl of 10% (w/v) fixed staphylococcus aureus for 40 min at 30 °C and centrifuged for 30 min at 50,000 x g. Supernatants were incubated with 10 μl of monospecific antibodies for 1 h at 30 °C before the addition of 50 μl of fixed staphylococcus aureus. After incubation for 20 min at 30 °C, the immunocomplexes were sedimented, washed five times with 0.1A SDS/0.1A Triton X-100/25M EDTA/10 mM Tris-HCl (pH 7.5) (buffer C) containing 1 M NaCl and 5.1A sodium lauroyl sarcosinate, and five times with buffer C. The pellets were treated for 3 min at 100 °C with 3A SDS/5% 2-mercaptoethanol/2 mM EDTA/50 mM Tris-HCl (pH 6.8)/1/A4 glycerol and then centrifuged. The supernatants were analyzed by electrophoresis in a 10% polyacrylamide gel containing SDS (2%). Radioactive bands were detected by fluorography using ENHANCE on Kodak XAR-5 film.

Immunoblot Analysis - Proteins in SDS gel after electrophoresis were transferred to a nitrocellulose sheet (0.45 μm) by electrophoresis, using a modification of the procedure of Towbin et al. (29). After transfer, the sheets were soaked for 1 h in 10 mM Tris-HCl (pH 7.5)/0.3 M NaCl/0.05% Triton X-100 (TBST) containing 1% bovine serum albumin (BSA), then incubated for 1 h with TBST containing 1% rat serum to avoid nonspecific adsorption, and thereafter, were incubated for 3 h with TBST containing the monospecific antibodies and 1% BSA. The sheets were washed 5 times with TBST and incubated for 1 h with TBST containing 1% BSA and goat anti-rabbit IgG conjugated with horseradish peroxidase, according to Nakane and Kawa01 (30). After 5 washings with TBST, the immunoreactive bands were detected by incubation with 0.02% 4-chloro-1-naphthol/0.01% hydrogen peroxide/50 mM Tris-HCl (pH 7.5).

Preparation and Labelling of Cultured Hepatocytes - Hepatocytes isolated from male Wistar rats (7 weeks old) by the collagenase perfusion method of Seglen (31) were diluted with Eagle's minimal essential medium containing 10% fetal calf serum, placed in 60 mm Falcon Tissue Culture Dishes (3 x 104 cells/dish) and then incubated in humidified air with 5% CO2 at 37 °C for 24 h. At this time, the cell monolayers were washed twice in Hank's solution supplemented with vitamins, 5% dialyzed fetal calf serum and amino acids, except for methionine, and incubated with 1 ml of this medium containing 100 μCi of [14C]methionine. The cell monolayers were washed once in Eagle's minimal essential medium containing 5% fetal calf serum and 2 mM methionine, and incubated in this medium until solubilized. After rinsing, media were removed and cells were lysed in 0.4 ml of lysis buffer. Immunoprecipitation was carried out as described in that section.

Endo γ-N-acetylgalactosaminidase H Digestion - Isolated immunocomplexes were digested by incubation with 0.1 μl of 2 μg/ml endo γ-N-acetylgalactosaminidase H (lot 5.0) at 37 °C for 30 min or by boiling for 3 min and then centrifuged. The supernatant was diluted with 0.9 ml of 2% Triton X-100/50 mM acetate buffer (pH 5.0)/protease inhibitors (10 μg/ml), thereafter, 5 μl of endo γ-N-acetylgalactosaminidase H was added. Following the incubation for 16 h at 37 °C, the mixtures were precipitated by the addition of 0.1 ml of 100% (w/v) trichloroacetic acid. The pellets were washed with acetone and analyzed on SDS gel electrophoresis.