Isolation and Characterization of a Novel Membrane Glycoprotein of 85000 Molecular Weight from Rat Liver Lysosomes

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We have purified and characterized a novel glycoprotein (r-lamp-3) with an apparent molecular weight (M_r) of 85000 from membranes of triton-filled lysosomes (tritosomes) by the use of immunoaffinity chromatography on a column of monoclonal antibody-Sepharose 4B. r-lamp-3 accounted for approximately 4% of the total proteins in tritosomal membranes. The isoelectric point (pI) of r-lamp-3 was 4.5 and it was shifted to 6.5 after neuraminidase treatment with its molecular weight decreased by about 7000. Pulse-chase experiments in cultured rat hepatocytes using [^{35}S]methionine showed that r-lamp-3 was initially synthesized as a 77000 polypeptide and processed to a mature protein with an M_r of 85000. Upon treatment with endo-β-*N*-acetylglucosaminidase H (Endo H), the precursor and mature forms were converted to 55000 and 73000 polypeptides, respectively. From the M_r reduction of the precursor form, we estimated the presence of 10—12 *N*-linked oligosaccharides/r-lamp-3 polypeptide. The data on enzymatic deglycosylation suggested that the mature form of r-lamp-3 contained the same numbers of high mannose-type and complex-type *N*-linked oligosaccharide chains.

Keywords glycoprotein; lysosomal membrane; monoclonal antibody; rat liver; cultured hepatocyte

Introduction

Recently, there have been increasing numbers of reports on the identification and characterization of lysosomal membrane glycoproteins taken from various cells and species.[1-9] These glycoproteins bear large numbers of *N*-linked sialylated complex-type carbohydrate chains with apparent molecular weights ranging from 100000 to 130000. Analysis on the sequence of complementary deoxyribonucleic acid (cDNA) has revealed that all the glycoproteins examined so far belong to two categories of glycoproteins.[10-18] We have purified the two kinds of glycoproteins designated as r-lamp-1 and r-lamp-2 from rat liver lysosomes.[6,7] Both the glycoproteins contained more than 50% carbohydrates. All the saccharides in r-lamp-1 were *N*-linked complex-type chains of which approximately 70% are sialylated, but r-lamp-2 possessed a small amount of *O*-linked oligosaccharide chains. Additionally, it was found that r-lamp-1 circulated continuously between the cell surface and lysosomes *via* the endocytic system in cultured hepatocytes.[8,9]

In this study, we purified and characterized a novel glycoprotein with M_r=85000 (r-lamp-3) from lysosomal membranes of rat livers. Pulse chase experiments showed that r-lamp-3 was highly glycosylated with high mannose-type and sialyl complex-type *N*-linked oligosaccharides.

Materials and Methods

Materials Male Wistar rats and male BALB/c mice were obtained from Shimizu Experimental Animal Co. (Kyoto, Japan). L-[^{35}S]Methionine was purchased from ARC (U.S.A.). Neuraminidase (*Anthrobacter ureafaciens*) was obtained from Nacalai Tesque (Kyoto, Japan). Endo-β-*N*-acetylglucosaminidase H (Endo H) (*Streptomyces griseus*) was from Seikagaku Kogyo Co. (Tokyo, Japan). All other chemicals were of reagent grade, and were purchased from various commercial sources.

Triton-Filled Lysosomes (Tritosomes) Male Wistar rats were injected intraperitoneally with Triton WR 1339 (85 mg/100 g body weight) 84 h before decapitation. Tritosomes were isolated from livers of the rats according to the method of Leighton *et al.*[19] Tritosomal membranes were prepared by an osmotic treatment as described.[6]

Preparation of a Monoclonal Antibody Hybridoma cells were developed by the fusion of murine myeloma (P3U1) and splenocytes obtained from mice immunized with the tritosomal membranes according to the method of Kohler and Milstein.[20] Positive clones were selected by immunoblot procedures with a Miniblotter 28 apparatus (Immunetics, Cambridge, MA) using the tritosomal membrane as a probe. Among the several positive clones, one clone, which produced an antibody for a protein with M_r=85000, was propagated and the antibody was produced as ascites in mice. This antibody, designated as mAb30 (IgG_1) was purified by chromatography on protein A-Sepharose CL 4B and coupled to BrCN-activated Sepharose 4B as described previously.[6]

Purification of r-lamp-3 The tritosomal membranes were suspended in 1% Lubrol PX (Sigma, St. Louis, MO) in 20 mM Tris–HCl (pH 7.0) at 4 °C for 30 min, and then centrifuged at 105000×*g* for 60 min. The resultant supernatants were applied to a column of mAb30-Sepharose 4B equilibrated with the solubilization buffer. The column was washed extensively with 20 mM Tris–HCl (pH 7.0) containing 0.1% Lubrol PX and 0.15 M NaCl, and then the bound materials were eluted with 6 M urea/20 mM Tris–HCl (pH 7.0)/0.1% Lubrol PX/1 mM phenylmethylsulfonyl fluoride/1 μM leupeptin. The eluted fraction was immediately concentrated by a Diaflo apparatus (Amicon, U.S.A.), and dialyzed against 20 mM Tris–HCl (pH 7.0) containing 0.05% Lubrol PX.

Gel Electrophoresis Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% slab gels according to Laemmli,[21] and two dimensional polyacrylamide gel electrophoresis according to O'Farrell[22] with nonequilibrium pH (3—10)-gradient electrophoresis for the first dimension. Proteins were stained with Coomassie Blue R-250.

Immunoblot Procedures Proteins in SDS-slab gel were transferred to a nylon membrane by electrophoresis. Positive bands were detected by using a modification of the procedure of Towbin *et al.*[23]

Protein Determination Proteins were determined by the method of Lowry *et al.*[24] using bovine serum albumin (BSA) as a standard.

Amino Acid Composition The purified protein was dialyzed against several changes of distilled water, lyophilized, and then hydrolyzed in 4 N methane sulfonic acid containing 0.2% 3-(2-aminoethyl)indole, under vacuum, for 24, 48, 72, and 96 h at 118 °C. Amino acids were analyzed by a Hitachi model 835 automatic amino acid analyzer.

Labeling of Cultured Hepatocytes and Immunoprecipitation Hepatocytes were obtained from male Wistar rats (200 g) using the collagenase-perfusion procedure originally described by Seglen.[25] Hepatocytes (3×10^6) were diluted with Eagle's essential medium containing 10% fetal calf serum, washed with Hanks' solution and then incubated in humidified air with 5% CO_2 at 37 °C for 24 h. At this time, the cell monolayers were washed twice in Hanks' solution supplemented with vitamins, 5% fetal calf serum and amino acids except for methionine, and then incubated for 15 min with this medium containing 100 μCi of [^{35}S]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 solubilization. After chasing, the media were removed and cells were lysed in 0.2 ml of 1% SDS/0.5% Lubrol PX/0.15 M NaCl/2 mM ethylenediaminetetraacetic acid (EDTA)/10 mM Tris–HCl (pH 7.0). After centrifugation at 10000 × *g* for 60 min, the resultant supernatant was diluted 10 times with 0.5% Lubrol PX/0.15 M NaCl/2 mM EDTA/10 mM Tris–HCl (pH 7.0)/protease inhibitors (leupeptin, pepstatin A, chymostatin and antipain, 10 μg/ml each). The lysates were preincubated with nonimmune IgG-Sepharose 4B for 8h at 4 °C followed by centrifugation. The supernatants were incubated with mAb30-Sepharose 4B for 18 h at 4 °C. The beads sedimented were washed five times with 1% Lubrol PX/0.5% deoxycholate/0.15 M NaCl/2 mM EDTA/0.1% BSA/10 mM Tris–HCl (pH 7.0) (buffer A), and five times with buffer A containing 2 M KCl and then twice with 0.1% SDS/0.5% Lubrol PX/0.15 M NaCl/10 mM Tris–HCl (pH 8.6). The immunocomplexes were dissociated with 0.1 ml of 2% SDS/50 mM acetate buffer (pH 5.0) by boiling for 3 min, then centrifuged. The supernatants were precipitated by adding 20 μl of 50% (w/v) trichloroacetic acid. The pellet was washed with ice-cold acetone and subjected to SDS-PAGE. Radioactive bands were detected by fluorography using EN^{3}HANCE on Kodak XAR-5 film.

Endo H Digestion Isolated immunocomplexes were dissociated in 2% SDS/50 mM acetate buffer (pH 5.0) by boiling for 3 min, then centrifuged. The supernatant was diluted 10 times with 2% Lubrol PX/50 mM acetate buffer (pH 5.0)/protease inhibitors (10 μg/ml); thereafter, 5 mU of Endo H was added. The mixtures were incubated for 18 h at 37 °C and then precipitated with 10% trichloroacetic acid. The pellets were washed with acetone and analyzed on SDS-PAGE.

Results and Discussion

One Step Purification of r-lamp-3 from Tritosomal Membranes When the tritosomal membranes were electrophoresed on SDS-polyacrylamide gel, a glycoprotein with M_r of 85000 (r-lamp-3) was conspicuously detected under the positions of r-lamp-1 (M_r = 107000) and r-lamp-2 (M_r = 96000).[6] The 85000 glycoprotein, designated as r-lamp-3 was purified from a Lubrol PX-solubilized fraction of tritosomal membranes by using immunoaffinity chromatography on an mAb30-Sepharose column. When a bound fraction was analyzed on SDS-PAGE, only one polypeptide band was detected in a position of M_r = 85000 (Fig. 1). The protein yield of the purified r-lamp-3 accounted for approximately 4.0% of the total proteins in tritosomal membranes (Table I). The contents of r-lamp-3 in tritosomal membranes was compatible with those of r-lamp-1 and r-lamp-2.[6,7]

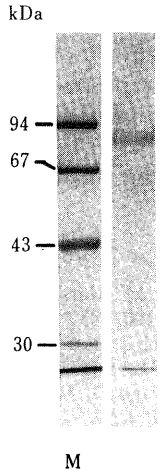

Fig. 1. SDS-PAGE of Immunoaffinity Chromatography Purified r-lamp-3

r-lamp-3 obtained from immunoaffinity chromatography of the Lubrol PX lysate of tritosomal membranes was analyzed by SDS-PAGE. The gels were stained with Coomassie blue for proteins. One microgram of the glycoprotein was electrophoresed on 10% acrylamide gel. Numerals indicated are the molecular sizes (kilodaltons (kDa)) of marker proteins (lane M) as follows: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa.

TABLE I. Purification of r-lamp-3 from Rat Liver

Step	Protein (mg)	Yield (%)
Homogenate	6800	100
Tritosomes	18.8	0.27
Tritosomal membranes	4.8	0.07
1% Lubrol PX lysate	3.5	0.05
Immunoaffinity chromatography	0.19	0.003

Approximately 40 g wet weight livers were used for the experiment.

TABLE II. Amino Acid Composition of r-lamp-3

Amino acid	mol%
Asx	11.9
Thr	6.0
Ser	8.4
Glx	9.5
Gly	10.1
Ala	7.3
Cys	2.4
Val	5.8
Met	1.1
Ile	5.4
Leu	8.8
Tyr	2.9
Phe	4.5
Lys	4.5
His	1.3
Trp	1.0
Arg	5.1
Pro	3.6
Total	99.6

Amino Acid Composition of r-lamp-3 The amino acid composition of the purified r-lamp-3 is shown in Table II. r-lamp-3 contained relatively high amounts of Gly, Leu and Ser. An attempt to determine the amino acid sequence of the *N*-terminus of r-lamp-3 by Edman degradation was unsuccessful, suggesting that the *N*-terminus may have been blocked (data not shown).

Neuraminidase Treatment r-lamp-3 before and after the neuraminidase digestion was subjected to two-dimensional gel electrophoresis (Fig. 2A). Non-treated r-lamp-3 was localized in M_r = 85000 and of pH 4.5. Upon treatment with neuraminidase, the glycoprotein moved to a position of M_r = 78000 and of pH 6.5. The decrease in M_r by 7000 was also shown in Fig. 2B. These data indicated that r-lamp-3 contained 8.3% sialic acid and was highly acidic, in common with r-lamp-1 and r-lamp-2.[6,7]

Pulse-Chase Analysis of r-lamp-3 in Cultured Primary Hepatocytes Cultured rat hepatocytes were pulse-labeled with [^{35}S]methionine for 15 min and subsequently chased up to 3 h in a medium containing an excess amount of unlabeled methionine. At definite times, r-lamp-3 was recovered by immunoprecipitation from the cell extracts. As shown in Fig. 3 (lane 2), at 15 min, r-lamp-3 was obtained as a polypeptide with M_r = 77000. During the following 30 min chase (Fig. 3, lane 4), the 77000 polypeptide was converted to a mature form with an M_r of 85000. After 1 h of chase (Fig. 3, lane 6), the mature form was a predominant band.

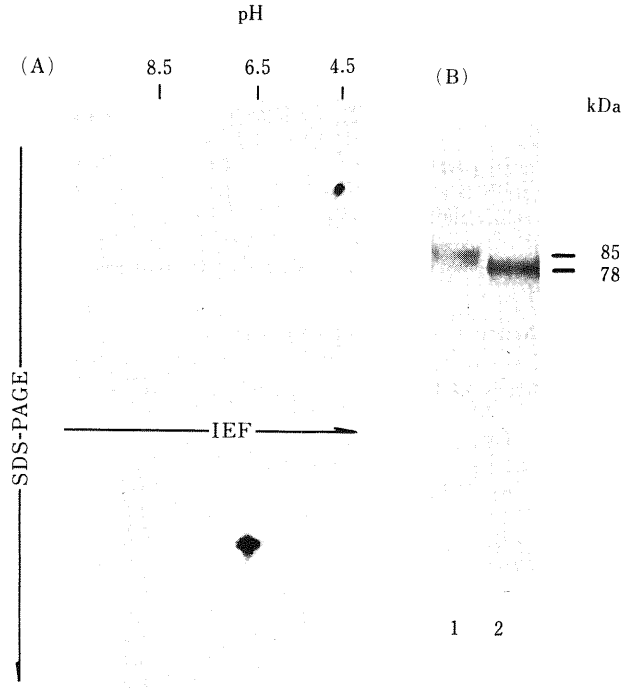

Fig. 2. Two-Dimensional Gel Electrophoresis and SDS-PAGE of r-lamp-3 before and after Neuraminidase Treatment

The purified r-lamp-3 (1 μg) was incubated with 5 mU neuraminidase at 37 °C for 1 h in 50 mM sodium acetate buffer (pH 5.0) containing 0.1% Lubrol PX. r-lamp-3 before and after the neuraminidase treatment was applied to two-dimensional gel electrophoresis (A) or SDS-PAGE (B). The gels were subsequently subjected to immunoblotting. (A) The upper panel: non-treated r-lamp-3, and the lower panel: neuraminidase treated r-lamp-3. (B) Lane 1; non-treated r-lamp-3, and lane 2: neuraminidase treated r-lamp-3.

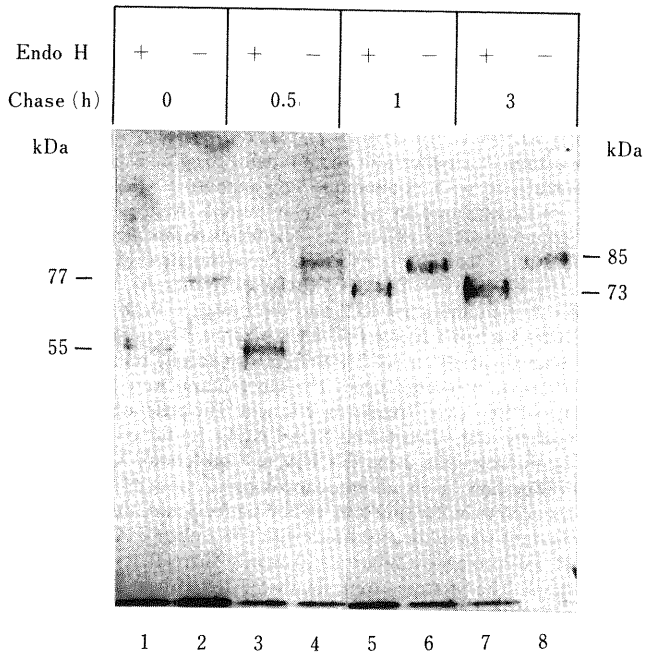

Fig. 3. Endo H Digestion of Precursor and Mature Forms of r-lamp-3

Cultured primary hepatocytes were pulsed with [^{35}S]-methionine for 15 min (lanes 1 and 2) or chased for 0.5 h (lanes 3 and 4), for 1 h (lanes 5 and 6) and for 3 h (lanes 7 and 8). r-lamp-3 was isolated from cell lysates by immunoprecipitation using mAb 30-Sepharose as described under "Materials and Methods." The glycoproteins were incubated with Endo H (lanes 1, 3, 5, and 7) or without Endo H (lanes 2, 4, 6, and 8). Samples were analyzed on SDS-PAGE followed by fluorography. Numerals on both sides indicate molecular masses (kDa) of polypeptides.

In order to characterize the carbohydrate moiety of r-lamp-3, the immature and mature forms of r-lamp-3 were digested with Endo H, an enzyme which cleaves the glycosidic bonds between the core *N*-acetylglucosamines of the high mannose-type *N*-glycan.[26] Upon the treatment with Endo H, the 77000 and 85000 polypeptides were converted to 55000 and 73000 proteins, respectively. From the difference in M_r between the immature forms before and after Endo H treatment, we estimated the presence of 10—12 *N*-linked oligosaccharides per mole of r-lamp-3, assuming an M_r value of 2000/high mannose-type oligosaccharide. Deglycosylation of the mature form with Endo H caused a reduction of 12000 in M_r, suggesting that mature r-lamp-3 contained approximately 6 high mannose-type *N*-linked oligosaccharide chains. The rest of the *N*-linked oligosaccharide chains seem to be a sialyl complex-type. Unlike r-lamp-3, almost all of the *N*-linked saccharides of r-lamp-1 were complex-type.[6] The chemical analysis data suggested that r-lamp-3 contained trace amounts of *O*-linked oligosaccharide chains: r-lamp-3 possesses only 3 moles of *N*-acetylgalactosamine residues per mol.

Groze *et al.* reported a unique lysosomal membrane glycoprotein with $M_r = 78000$ from rat cells.[27] The glycoprotein resides not only in lysosomes but also in endosomes, thus referred to as endolyn-78. Endolyn-78 is highly glycosylated with both *N*- and *O*-glycans. Endolyn-78 is different from r-lamp-3 in that the amount of *O*-glycans in endolyn-78 are much larger than those in r-lamp-3. Unlike r-lamp-3, [^{35}S]methionine was not incorporated into the endolyn-78 molecules.

The present pulse-chase experiments suggested that r-lamp-3 follows a biosynthetic pathway analogous to the other lysosomal membrane glycoproteins.[4] In rough endoplasmic reticulum, r-lamp-3 in first synthesized as a glycosylated precursor with $M_r = 77000$ containing 10—12 high mannose-type oligosaccharides. Half of the high mannose-type *N*-glycans in r-lamp-3 are processed to complex-type during passage through the Golgi apparatus. Then, r-lamp-3 is transported to the lysosomes. The mechanisms for the sorting of r-lamp-3 to lysosomes remain unknown at present. Williams and Fukuda recently identified a signal for the delivery of h-lamp-1 to lysosomes as a tyrosine residue at a particular position in the short cytoplasmic tail of the h-lamp-1 polypeptide.[28] Cloning of cDNA corresponding to r-lamp-3 is prerequisite for understanding the sorting signal and function peculiar to this glycoprotein.

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