THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 14, Issue of May 15, pp. 10207-10213, 1993
Printed in U.S.A.

Structural, Immunological, and Biosynthetic Studies of a Sialic Acid-specific *O*-Acetylesterase from Rat Liver*

(Received for publication, November 23, 1992, and in revised form, February 2, 1993)

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We have previously described a membrane-associated intralumenal sialic acid-specific 9-*O*-acetylesterase (LSE) from rat liver (Higa, H. H., Manzi, A., and Varki, A. (1989) *J. Biol. Chem.* 264, 19435–19442). Unlike a cytosolic sialate:*O*-acetylesterase (CSE) with similar specificity, the LSE carries *N*-linked oligosaccharides. A polyclonal monospecific antibody against homogenous LSE does not cross-react with the CSE. Monoclonal antibodies distinguish between the LSE and another *N*-glycosylated esterase that tends to partially co-purify with it. Amino-terminal sequencing of the LSE subunits indicates that it is distinct from previously described esterases and shows no homology to any other known proteins. In contrast, the esterase that partially co-purifies is similar but not identical to previously described "microsomal" esterases from rat liver.

The LSE is also expressed in several hepatoma cell lines. Pulse-chase studies indicate that the two LSE subunits arise from a single precursor of ~65 kDa which yields a core polypeptide of apparent molecular mass ~53 kDa upon deglycosylation with peptide:*N*-glycosidase F. The protein quickly becomes partly resistant to endo-β-*N*-acetylglucosaminidase H but remains sensitive to peptide:*N*-glycosidase F, indicating *N*-linked oligosaccharide processing during passage through the Golgi. After several hours, the precursor undergoes proteolysis, generating the mature heterodimeric protein of ~58 kDa, with subunits of ~38 and ~28 kDa. A portion of newly synthesized LSE is secreted into the medium intact, indicating that the cleavage normally takes place after diversion from the secretory pathway. These temporal changes and precursor-product distribution are reminiscent of some lysosomal acid hydrolases. In fact, immunofluorescence studies and Triton WR-1339 shift experiments suggest a lysosomal localization for this enzyme. Additional evidence for this, and the role of the LSE in *O*-acetylated sialic acid turnover are discussed in the accompanying paper (Butor, C., Diaz, S., and Varki, A. (1993) *J. Biol. Chem.* 268, 10197–10206).

The sialic acids are a diverse family of acidic nine-carbon sugars that are frequently found as terminal units of oligosaccharide chains on different glycoconjugates (1–3). Many natural modifications of sialic acids have been reported, all of which are believed to arise from the parent compound *N*-acetylneuraminic acid (Neu5Ac)[1] (1–4). Modifications of the sialic acids show tissue-specific and developmentally regulated expression and appear to modulate a wide variety of biological phenomena (2, 3). The mechanisms regulating the expression and turnover of these modified sialic acids are therefore of considerable interest.

We have previously described *O*-acetylation of the side chain of sialic acids in rat liver Golgi-enriched preparations and predicted the existence of multiple sialic acid-specific *O*-acetylesterases in this tissue (5–7). Currently available evidence indicates the presence of at least two such activities: a *cytosolic* sialate, 9-*O*-acetylesterase (CSE), and a membrane-associated intralumenal sialate:9-*O*-acetylesterase (LSE). The latter is an *N*-linked glycoprotein that remains associated with membrane-limited compartments after gentle tissue homogenization. In previous work, we described the purification of this enzyme to homogeneity (6). The homogeneous LSE from rat liver is a 62-kDa disulfide-linked heterodimeric protein, with a serine active site that can be labeled by [^{3}H]DFP. Two subunits of 36 and 30 kDa are generated upon reduction, and the 30-kDa subunit carries the [^{3}H]DFP label and presumably the active site serine. The protein is cleaved by peptide:*N*-glycosidase F (peptide:*N*-glycosidase F) and to a lesser extent by endo-β-*N*-acetylglucosaminidase H (endo H), indicating that it has both high mannose-type and complex-type *N*-linked sugar chains. Studies with various substrates show that the enzyme is specific for sialic acids and selectively cleaves *O*-acetyl groups only from the 9-position (6).

In the accompanying paper (7), we studied the expression and biosynthesis of *O*-acetyl groups on sialic acids in rat liver membranes and showed that the LSE was predominantly associated with lysosomal markers during subcellular fractionation. In the current study, we present new structural, immunological, and biosynthetic data concerning this enzyme.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: resins AG50W-X2 (H^+ form) and AG 3X4A (formate form),

* This research was supported by a Merit Review Award from the Veterans Administration Research Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; LSE, intralumenal (lysosomal) sialate:*O*-acetylesterase; CSE, cytosolic sialate:*O*-acetylesterase; endo H, endo-β-*N*-acetylglycosaminidase H; DFP, diisopropylfluorophosphate; ELISA, enzyme-linked immunosorbent assay; 4MU-acetate, 4-methylumbelliferyl acetate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ER, endoplasmic reticulum.

Bio-Rad; *Arthrobacter ureafaciens* sialidase, Calbiochem; protein molecular weight standards, 4-methylumbelliferyl acetate (4MU-acetate), Sigma; and endo H, Miles. DFP from Aldrich was prepared as a 100 mM stock solution in isopropyl alcohol stored at −20 °C. All other chemicals were of reagent grade. Peptide:N-glycosidase (8) was purified from the culture supernatant of *Flavobacterium meningosepticum* and shown to be protease-free as described previously (9). [^{3}H] DFP (5.8 Ci/mmol) was from Amersham Corp., and a mixture of labeled cysteine and methionine (Expre^{35}S^{35}S) was from Du Pont-New England Nuclear.

Purification of Homogeneous LSE from Rat Liver—The LSE and the 66-kDa esterase that tends to co-purify with it were purified and separated as described previously (6), with some modifications. The enzyme was purified from fresh frozen rat livers, rather than from rat liver acetone powder. Rat livers (purchased from Pel-Freez Biologicals) were finely minced while still frozen and homogenized with a Polytron in 20 mM KP_i, pH 8.0. The LSE was released from the crude liver homogenates by five cycles of freeze-thaw and recovered by passing the mixture over cheesecloth and centrifuging at 165,000 × *g* for 30 min. The supernatant fluid was collected and pooled. Each pellet was resuspended in 10 ml of the same buffer and recentrifuged as above. The pooled supernatants were passed over glass wool and then loaded onto the DE52-cellulose DEAE column as described previously (6). From this step, the purification procedure was identical to that previously described, except for a slight change in the final procion red step. We found that the procion red needs to be put through one regeneration cycle following the manufacturer's instructions, before it will allow the previously described esterase purification. Also, we found that the procion red shows some batch-to-batch variability in the exact salt concentration required for elution and that it does not perform identically upon repeated re-use.

SDS-PAGE Analysis of Proteins—These were performed in a standard fashion (10), using 8–10% gels. The gels were fixed, stained with Coomassie Blue if necessary, impregnated with Intensify™ (Du Pont-New England Nuclear), dried, and subjected to fluorography.

Amino-terminal Sequencing of Proteins—Purified preparations of the enzyme were reduced and denatured, separated by SDS-PAGE, transferred to polyvinylidene difluoride (Immobilon) filters, stained with Coomassie Blue, the individual bands cut out, and submitted for amino-terminal gas phase sequencing at the University of California, San Diego Peptide Sequencing Facility.

[^{3}H]DFP Labeling of Esterases—The purified esterases were labeled individually or in mixtures by adding 1 μl of [^{3}H]DFP to 100 μl of the enzyme prep in PBS, incubating on ice for 30–60 min, and dialyzing against three changes of 500 ml of PBS.

Raising Polyclonal Monospecific Antibodies against the LSE—Polyclonal antibodies were raised in New Zealand White male rabbits, following a standard protocol (11). Primary immunization was performed with 100–160 μg of purified sialic acid esterase intradermally in complete Freund's adjuvant. Boosts (25–80 μg) in incomplete Freund's adjuvant were performed subcutaneously. On some occasions the esterase was gel-purified before injection. Bleeds were performed via the ear vein.

Raising Monoclonal Monospecific Antibodies against the LSE—Monoclonal antibodies were generated by following a standard protocol (12). Swiss Webster mice were immunized with a preparation containing roughly equal amounts the LSE and the co-purifying ("contaminating") esterase. The mouse giving the best antibody response was sacrificed and spleen cells fused with the NS-1 partner by the hybridoma facility of the University of California, San Diego Cancer Center. The initial screening was performed by ELISA against material from the S-200 purification step (6). Positive clones were then screened by immunoprecipitation of [^{3}H]DFP-labeled immunogen using a polyclonal rabbit anti-mouse immunoglobulin (Dakopatts) as bridging antibody. Selected clones directed against the sialic acid esterase or the contaminating esterase were then subcloned by limiting dilution and screened again by immunoprecipitation. Clones were maintained in RPMI with 10% fetal calf serum. Conditioned medium was concentrated by ammonium sulfate precipitation and the concentrate stored frozen at −20 °C.

Immunoprecipitation of Labeled LSE from Cells—FAO cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12, 1:1, with 2 mM L-glutamine and 10% fetal calf serum. For [^{35}S]Met/Cys labeling, the cells were grown for 3 days in 35-mm Petri dishes or in 12-well plates. The cells were placed in methionine-free Eagle's modified essential medium for 10–30 min. They were then labeled for 15 or 60 min with 0.2–1 mCi of Expre^{35}S^{35}S in 200–500 μl of methionine-free Eagle's modified essential medium. The labeled medium was then replaced with standard growth medium for 15 min to 96 h. In some experiments, the cells were labeled continuously for various periods of time, in complete medium. The cells were washed and scraped in ice-cold PBS and spun down in a tissue-culture centrifuge at 4 °C. The cell pellet was solubilized in 100–200 μl of lysis buffer (1% Nonidet P-40, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 μg/ml phenylmethylsulfonyl fluoride) by passing it five times through a 22-gauge needle. The lysate was spun for 5 min in a Beckman Microfuge and the supernatant frozen at −80 °C until further use. The supernatant (one-half to two-thirds of the volume) required extensive preclearing (two 24-h incubations with protein A-Sepharose beads and three with protein A beads coated with 1/100 to 1/1000 dilutions of preimmune serum). The same bleed from one rabbit that produced a monospecific antibody was used for all pulse-chase experiments presented in this paper. The precleared lysate was incubated for 1–2 h with protein A beads coated with the immune serum, appropriately diluted. The beads were then washed six to eight times. Three washes (one of which was overnight) were performed with 50 mM Tris/HCl, pH 8.0, 1.2 M KCl, 1.2% Triton X-100 (13). This washing solution considerably reduced the background of nonspecific proteins in the immunoprecipitate. The rest of the washes were performed with 50 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 0.5%, v/v, Nonidet P-40, 0.04% SDS, 0.5%, w/v, deoxycholic acid (preclear buffer). The washed pellets were stored frozen at −80 °C. For enzymatic treatments, they were dissolved in 0.2% SDS, 10 mM Tris/HCl, pH 8.0. The immunoprecipitates were split into three aliquots. One was treated with peptide:*N*-glycosidase F and one with endo H as described previously (6, 8), before running on SDS-PAGE gels. Unless otherwise stated, the pellets were boiled in 2 × Laemmli sample buffer without β-mercaptoethanol. Half of each sample was then reduced with 10 mM dithiothreitol and acetylated with 20 mM iodoacetamide. Reduced and nonreduced samples were run in parallel on SDS-PAGE gels.

Immunoprecipitation of [^{35}S]Met/Cys-labeled cell media, column eluates, and [^{3}H]DFP-labeled samples did not require this extensive preclearing. Only one preclearing with plain protein A beads was necessary, and all washes could be performed with the preclear buffer.

Immunofluorescent Studies of the LSE in Permeabilized FAO Hepatoma Cells—FAO cells were grown on glass slides, washed in PBS, and fixed for 20 min in 3% formalin in PBS. The slides were washed again in PBS and the free aldehyde groups quenched for 10 min in 50 mM NH_4Cl in PBS. The fixed cells were permeabilized for 5 min in PBS with 0.1% Triton X-100 and washed. They were then incubated with primary antibody (immune or preimmune) appropriately diluted in PBS containing 0.2% cold water fish skin gelatin (w/v) for 30 min in a humid chamber. The cells were washed and incubated with fluorescein-labeled goat-anti rabbit or rabbit anti-mouse antibodies as appropriate. After final washes, the cells were mounted in Gelmount (Biomeda) with a coverslip. Slides were observed in a Zeiss photomicroscope equipped for epifluorescence.

Triton TWR 1339-induced Shift of Lysosomes on Sucrose Gradients—14-week-old Sprague-Dawley rats were injected with Triton TWR 1339 according to Ref. 14 or left untreated as controls. Livers were homogenized in 0.5 M sucrose, 50 mM sodium maleate, 5 mM $MgCl_2$, pH 6.5. After low speed centrifugation to clear nuclei and debris, the homogenate was layered on a 0.5–1.5 M continuous sucrose gradient in the same buffer and centrifuged for 14 h at 75,000 × *g*. Fractions were collected from the bottom of the gradient and assayed for 9-*O*-acetylesterase activity and for various other marker enzymes.

Mannose 6-Phosphate Receptor Affinity Chromatography—The cation-dependent mannose 6-phosphate receptor was purified from bovine liver acetone powder and immobilized on Affi-Gel-10 as described previously (15). All steps were carried out at 4 °C. Labeled cell lysates were diluted with PBS and spun in a Beckman Microfuge, and the supernatant was passed over a small Sepharose precolumn, prior to loading on the receptor column. After washing with ~20 column volumes, the column was eluted with 5 mM mannose 6-phosphate in PBS. Fractions were collected and the radioactivity monitored.

RESULTS

A Polyclonal Monospecific Antibody against the Purified LSE Does Not Cross-react with the CSE—Although the LSE and CSE of rat liver have similar substrate specificities, the CSE has so far not been purified to homogeneity. Thus, the possibility remains that the two activities represent the same

protein, present in two different locations. The availability of homogenous preparations of LSE (6) allowed us to see if there was immunological cross-reactivity between this enzyme and the CSE. Polyclonal antibodies against the purified LSE were raised in several rabbits as described under "Experimental Procedures." Because of trace contamination of the immunogen with another 4MU-acetate esterase of 66 kDa (6), only some rabbits developed antibodies that were monospecific for the LSE, whereas others gave antibodies against both protein bands. One monospecific antibody against LSE reacted only with the native form of the enzyme, whereas another reacted with both the native and denatured forms, detecting a single broad 61–62-kDa band on a Western blot of a nonreducing SDS-PAGE gel of a rat liver extract. To see if the CSE from rat liver was structurally related to the LSE, we studied how well the two enzymes competed for such polyclonal antibodies. Since the CSE was not purified to homogeneity, we used partially purified preparations of both enzymes for competition studies. The CSE preparation used was eluted with salt from the initial DEAE-cellulose column and passed thru ConA-Sepharose, whereas the LSE preparation ran through the DEAE column and was bound and eluted from the ConA column (6). A monospecific polyclonal antibody was preincubated with amounts of each partially purified preparation that had the same activity (20 units) against the 9-*O*-acetyl sialic acid substrate (6). Following preincubation with serial dilutions of the antibody, residual active antibody was assessed by a direct ELISA assay against the purified LSE. As shown in Fig. 1, the partially purified CSE did not compete at all for the antibody, whereas a similar number of units of the partially purified LSE competed very well. Assuming that the enzymes do not have markedly different specific activities, this experiment with a polyclonal antibody clearly indicates that the two activities are immunologically distinct from one another (if the enzymes do have widely disparate specific activities, this would also indicate that they must be very different). Of course, to confirm *complete* lack of cross-reactivity, similar studies must eventually be done with highly purified preparations of both enzymes and with several antibodies. Regardless, these data indicate that the LSE and CSE are two distinct proteins.

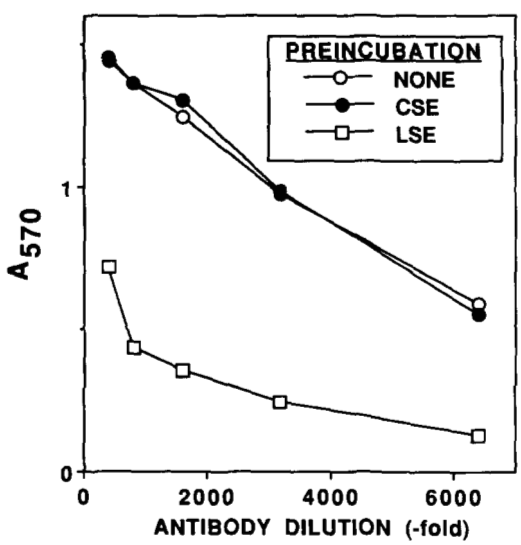

FIG. 1. **Competition of polyclonal antibody with esterase-containing fractions.** An ELISA against the homogenous rat liver LSE was set up using a polyclonal monospecific antibody, as described under "Experimental Procedures." Serial dilutions of the antibody (100 μl) were preincubated with partially purified preparations from rat liver (100 μl) containing either the CSE (DEAE-eluted peak, ConA run through) or the LSE (DEAE run thru, ConA eluate). The aliquots used for competition each contained the same amount of 9-*O*-acetylsialic acid esterase activity (20 units). Following preincubation at room temperature for 20 min, the mixtures were centrifuged at 8000 × *g* for 5 min, and 100-μl aliquots were transferred to microtiter wells for the ELISA, as described under "Experimental Procedures."

Monoclonal Antibodies against the LSE Do Not Cross-react with the Contaminating Esterase—During purification of the LSE to homogeneity, it was necessary to separate it from another protein with 4MU-acetate esterase activity that co-purified with it through several steps and tended to contaminate the final preparation (6). Like the LSE, the latter enzyme was water-soluble, bound to ConA-Sepharose, and had very similar molecular mass properties co-eluting in S-200 gel filtration chromatography and having only a slightly higher apparent molecular mass (66 kDa) on SDS-PAGE. Separation of the two enzymes was achieved only at the final procion red-agarose step, by differential salt elution (see Fig. 3 of Ref. 6). Although the other esterase shared the properties of 4MU-acetate cleavage and DFP sensitivity (indicating a serine active site mechanism), it did not have any activity against 9-*O*-acetylsialic acid (6). As mentioned above, many of the rabbit polyclonal antibodies raised against the LSE could precipitate both enzymes, presumably because the immunizing LSE preparations were contaminated with traces of the other esterase (hereafter called the contaminating esterase). To obtain alternate evidence that the latter enzyme was distinct from the LSE, we raised monoclonal antibodies. Mice were immunized with a mixture of the two enzymes (prior to final separation by procion red-agarose) and hybridoma supernatants were screened by ELISA for antibodies against the mixture. Positive clones were then studied for immunoprecipitation with a [^{3}H]DFP-labeled mixture of the two esterases. It was found that although four monoclonal antibodies specifically precipitated only the LSE, 16 others precipitated only the contaminating esterase and one immunoprecipitated both proteins (data not shown). These data indicate that although the proteins are immunologically quite distinct, they may share certain structural features, perhaps related to the fact that they are both DFP-sensitive serine active site enzymes.

Amino-terminal Sequences of Both Subunits of the LSE Indicate a Unique Protein, Distinct from Previously Described Esterases and from the Contaminating Esterase—To obtain further evidence that the LSE is distinct from previously described rat liver esterases, we obtained amino-terminal sequences from the two subunits. Purified preparations of the enzyme were reduced, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to amino-terminal gas phase sequencing. As shown in Table I, 28 residues of the small subunit and 30 residues of the large subunit were sequenced, with few ambiguities. In the case of the large subunit, three closely clustered but distinct Coomassie staining bands were seen in some preparations, especially if very long gels were run (data not shown). However, the amino-terminal sequences of all these forms were found to be identical (see Table I), indicating that they may result from differential glycosylation on a common polypeptide chain or possibly from differential cleavage at the carboxyl-terminal end. The amino-terminal sequences of the two subunits were compared with existing sequences in the Swiss-Prot and PIR data bases (Versions 23.0 and 34.0, respectively) and with amino acid sequences translated from open reading frames in GenBank version 73 (Genpept) using the FASTA and BLAST search programs. In addition, the sequences were kindly examined by Dr. R. Doolittle (University of California,

TABLE I

Amino-terminal amino acid sequences of rat liver esterases

Residues indicated with italics gave nonconclusive signals in sequencing.

	1			5				10				15				20				25				30
LSE small subunit	Ile	Pro	Phe	Pro	Phe	Ala	Ser	Tyr	Ile	Asp	Asn	Tyr	Met	Val	Leu	Gln	Lys	Glu	Pro	Ser	Gly	Ala	Val	Ile	*Leu*	Gly	Phe	Leu			
LSE large subunit 1	Gly	Pro	Ala	Thr	His	Ser	Val	Leu	*Trp*	Asn	Ala	Met	Ile	Pro	Leu	Gln	*Thr*	Met	*Arg*	Leu	Lys	Gly	Val	*Val*	*Trp*	Tyr	Gln	Gly	Glu	Asn	
LSE large subunit 2	Gly	Pro	Ala	Thr	His	Ser	Val	Leu	*Trp*	Asn																					
LSE large subunit 3	Gly	Pro	Ala	Thr	*His*	Ser	Val	Leu	*Trp*	Asn	Ala	Met	Ile																		
Contaminating esterase	Asp	Pro	Ser	Ser	Pro	Pro	Val	Val	Asp	*Thr*	*X*	Lys	Gly	Lys	Val																
RL1 (pI 6.5)[a]	Asp	Pro	Ser	*X*	Pro	Pro	Val	Val	Asp	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val	Ser	Leu									
RH1 (pI 6.0)[a]	Tyr	Pro	Ser	*X*	Pro	Pro	Val	Val	Asp	*X*	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val											
RL2 (pI 5.5)[a]	*X*	Pro	Ser	*X*	Pro	Pro	Val	Val	Asp	*X*	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val											
E1 a (pI 5.7)[b]	Tyr	Pro	Ser	Ser	Pro	Pro	Val	Val	Asn	Thr																					
E1 b[c]	His	Pro	Ser	Ser	Pro	Pro	Val	Val	Asp	Thr	Thr	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val	Ser	Leu	Glu	Gly	Phe	Thr	Gln	Pro	Val	Ala	
59 kDa cDNA[d]	His	Pro	Ser	Ser	Pro	Pro	Val	Val	Asp	Thr	Thr	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val	Ser	Leu	Glu	Gly	Phe	Thr	Gln	Pro	Val	ALa	
59-kDa peptide[d]	Tyr	Pro	Ser	Ser	Pro	Pro	Val	Val	Asn	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly		Tyr	Val	Asn	Leu	Glu	Gly	Phe						
ES-10 (pI 6.1)[e]	Tyr	Pro	Ser	Ser	Pro	Pro	Val	Val	Asn	Thr	Val	Lys	Gly	Lys	Val	Leu	Gly	Lys	Tyr	Val	Asn	Leu	Glu	Gly	Phe	Ala	Gln	Pro	Val	Ala	

[a] Hosokawa *et al.* (24) peptide sequences.
[b] Harano *et al.* (25) peptide sequence.
[c] Takagi *et al.* (26) cDNA sequence.
[d] Long *et al.* (27) peptide and cDNA sequences.
[e] Robbi *et al.* (28) cDNA sequence.

San Diego), using additional search criteria. No significant homologies were found to any of the sequences searched, indicating that the LSE is a unique hitherto unidentified protein.

The amino-terminal sequence of the contaminating esterase was also obtained. This is also shown in Table I, in comparison with the LSE sequences and with the amino-terminal sequences of a family of esterases previously reported by others in the rat liver (16–28) and in other mammalian species (29–34) (the latter are not shown in the table). It can be seen that the contaminating esterase is very distinct from the LSE but very similar to these previously described rat liver "microsomal" esterases that are believed to reside in the endoplasmic reticulum (ER). Thus, the esterase that partially co-purifies with the LSE is similar to this previously described gene family of ER esterases (perhaps identical with the pI 6.5 enzyme). In contrast, the LSE is evidently not a member of this family of enzymes.

The LSE Is Expressed in Hepatoma Cells, and the Two Subunits Arise from a Single Precursor Polypeptide—The two-disulfide bonded subunits of the mature LSE could arise either from two distinct precursor polypeptide chains that become associated or from the proteolytic cleavage of a single polypeptide chain. To study this issue, we examined several rat hepatoma cells for expression of the CSE and LSE (defined by ConA binding of 9-*O*-acetylesterase activity). We found that several such lines (FAO, MH_1C_1, HTC, and Ft02b) expressed both activities to varying degrees. Based upon the growth characteristics and the level of expression of the enzymes, we chose the FAO hepatoma cell line for further study of the LSE. A mono-specific rabbit polyclonal antibody that did not cross-react with the contaminating esterase was used to immunoprecipitate the labeled LSE from cell lysates during a pulse-chase analysis with [^{35}S]Met/Cys. As shown in Fig. 2, the LSE is initially synthesized as a single precursor polypeptide chain of ~65 kDa, which does not yield any subunits upon reduction. Pulse-chase analysis indicates that

FIG. 2. **Biosynthesis and processing of the LSE in FAO hepatoma cells.** Near-confluent cells were pulsed with [^{35}S]Met/Cys in methionine-free media for 1 h. One plate was harvested immediately, and the others were chased for varying periods of time in complete media, up to 48 h. Labeled cells were lysed and the LSE immunoprecipitated with a monospecific polyclonal antibody as described under "Experimental Procedures." The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The chase times (from *left* to *right*) are 0, 2, 4, 8, 16, 24, 36, and 48 h. Molecular mass markers are indicated on the *left*. The bands in the region marked with an *asterisk* were seen in control precipitates using preimmune serum and are not considered significant. The bands specific for the immune serum are indicated on the *right* by *open arrowheads*. The smaller subunit bands appearing in the reduced sample during the chase period are rather diffuse and difficult to photograph well.

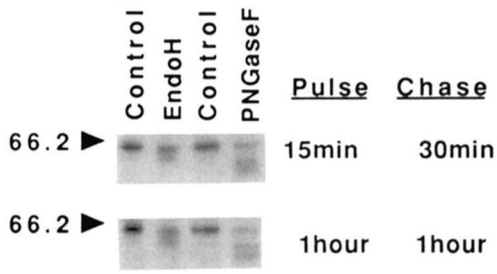

FIG. 3. **Analysis of *N*-linked oligosaccharides on LSE in FAO hepatoma cells.** Near-confluent cells were pulsed with [^{35}S] Met/Cys in methionine-free medium for 15 min or 1 h. One plate of each was harvested immediately, and others were chased for varying periods of time in complete medium. Labeled cells were lysed, the LSE immunoprecipitated with a monospecific polyclonal antibody as described under "Experimental Procedures," and aliquots of the immunoprecipitate treated with endo H or peptide:*N*-glycosidase F prior to SDS-PAGE separation and fluorography. Molecular mass are indicated on the *left*. Complete digestion with peptide:*N*-glycosidase F proved to be difficult to obtain, perhaps because the LSE protein is very difficult to denature.[2]

the precursor gradually undergoes a proteolytic cleavage after several hours, generating the mature heterodimeric protein of ~58 kDa, that upon reduction gives two subunits of ~38 and ~28 kDa. The onset of the proteolytic cleavage varied from 2 to 6 h in various experiments, with completion varying between 8 and 16 h. The diffuseness of the bands, particularly after reduction, was consistently seen in all experiments and is probably due to microheterogeneity in glycosylation. Although this makes it somewhat difficult to obtain good photographs, the original fluorographs were clear enough to allow assignments of the subunit molecular masses. The molecular masses obtained in these metabolically labeled hepatoma cells are similar but not identical to those seen for the LSE from normal rat liver. These differences may be due to variations in glycosylation.

The LSE Expressed in Hepatoma Cells Is a Glycoprotein Whose N-Linked Oligosaccharides Undergo Processing, Indicating Passage through the Golgi Apparatus—To examine the presence and nature of *N*-linked oligosaccharides on the LSE, the FAO cells were pulsed with [^{35}S]Met/Cys for 15 min or 1 h, chased for various periods of time, and the immunoprecipitates treated with endo H and peptide:*N*-glycosidase F prior to analysis. The single precursor polypeptide chain of ~65 kDa was sensitive to endo H and peptide:*N*-glycosidase F soon after synthesis, yielding a core polypeptide of ~52 kDa. This indicates the addition of high mannose-type *N*-linked oligosaccharides, presumably at the site of initial synthesis in the ER. Some examples of the results obtained are shown in Fig. 3. After a 15–60-min chase, the protein becomes partly resistant to endo H but remains susceptible to peptide:*N*-glycosidase F, indicating passage through the Golgi apparatus, where some *N*-linked oligosaccharides have been processed to complex-type chains (8, 35). As shown in the example in Fig. 3, complete digestion with peptide:*N*-glycosidase F was difficult to obtain. This may be related to the fact that the mature protein has proven to be very difficult to denature.[2] However, in all experiments, the lowest molecular mass seen after peptide:*N*-glycosidase F at early time points was 52 kDa. The intermediates seen suggest the presence of at least two *N*-linked glycosylation sites. Finally, the proteolytic cleavage that generates the mature heterodimeric protein occurs long after the partial conversion to endo H resistance. Thus, this cleavage may occur after the protein has completely passed through the Golgi apparatus.

A Secreted Form of the LSE Is Not Proteolytically Cleaved—A portion of the biosynthetically labeled LSE was also found secreted into the medium, appearing first about 2 h after the onset of labeling and accumulating thereafter (see Fig. 4 for a representative experiment). Unlike the intracellular material, this secreted protein is in the uncleaved form and does not change in molecular mass with reduction. This further indicates that proteolytic cleavage of the intracellular form must be a late event that takes place after the first passage of newly synthesized enzyme through the Golgi apparatus. Even after a long chase (96 h) the medium contains almost exclusively the uncleaved form (Fig. 4). Since the intracellular form is completely processed by this time, all of the protein found in the medium must arise from direct secretion of newly synthesized material.

Evidence for a Lysosomal Localization of the LSE—The kinetics of synthesis and proteolytic cleavage of the LSE are somewhat reminiscent of that seen for some lysosomal enzymes, such as cathepsin D (36–39). In the accompanying paper (7), we show that the sialate:9-*O*-acetylesterase activity corresponding to the LSE co-fractionates with lysosomal markers in both differential fractionation and continuous sucrose gradients. Using the monospecific polyclonal and monoclonal antibodies against the LSE with permeabilized and fixed FAO cells, immunofluorescence studies showed a punctate pattern typical of lysosomes (data not shown). The pattern was distinct from that seen with rhodamine 123 staining of mitochondria, which gave a speckled and partly filamentous pattern (data not shown). The only other subcellular organelles that could give such a punctate appearance are peroxisomes, secretory granules, and endosomes. The first two are unlikely based upon the data already presented for the biosynthesis of the enzyme. The data presented in the accompanying paper (7) indicate a co-fractionation of the LSE with lysosomal markers and co-distribution in the heavy part of sucrose gradients. Since endosomes would be expected to be lighter in such gradients, at least the bulk of the LSE cannot be in these organelles. However, since lysosomes have a notoriously variable sedimentation in such gradients, their

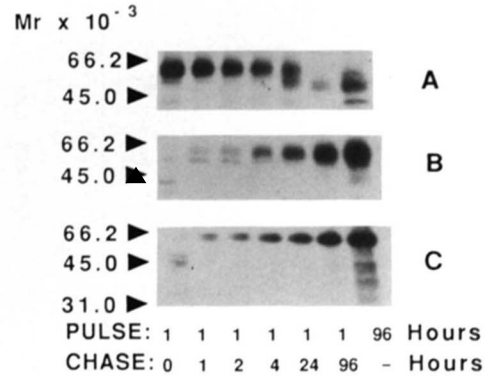

FIG. 4. **Secretion of newly synthesized LSE by FAO hepatoma cells.** Near-confluent cells were pulsed with [^{35}S]Met/Cys in methionine-free medium for 1 h. One plate was harvested immediately, and others were chased for varying periods of time, up to 96 h in complete medium. An additional plate was continuously labeled in complete medium for 96 h. [^{35}S]Met/Cys-labeled cell lysates and medium were immunoprecipitated with a monospecific polyclonal antibody against the LSE, as described under "Experimental Procedures." The immunoprecipitates were analyzed by SDS-PAGE and fluorography. *A*, from cellular material, unreduced; *B*, from medium, unreduced; and *C*, from medium, reduced. The pulse and chase times for individual samples are indicated in the figure.

[2] B. K. Hayes and A. Varki, unpublished observations.

identification is further improved by the density shift induced upon treating the animal with Triton TWR-1339 (14). In untreated rats, both β-hexosaminidase and LSE show very similar profiles in a continuous sucrose gradient (the esterase activity at the top of the gradient represents the cytosolic CSE). As shown in Fig. 5, TWR-1339 treatment of the rat causes both enzymes to show a nearly identical shift into lighter lysosomes. Taken together, these data indicate a mainly lysosomal localization for this enzyme in the rat liver.

Does the LSE Utilize the Man-6-P Receptor Pathway to Target to the Lysosomes?—Several lines of evidence presented in this paper and in the preceding one (7) suggest that although a fraction of the newly synthesized LSE is secreted, the majority is targeted to lysosomal compartments. It is well known that most, but not all, lysosomal enzymes utilize the mannose 6-phosphate receptor pathway for selective trafficking to the lysosome (40, 41). Although alternative pathways for lysosomal trafficking occur in some cell types, including the liver, phosphorylation of the enzymes still occurs in these cells (38, 40–43). To see if the LSE utilized this pathway, we asked if secretion of the enzyme was increased when the hepatoma cells were grown in the presence of 10 mM ammonium chloride, which causes enhanced secretion of newly synthesized lysosomal enzymes (44–46). However, under conditions where a 2–4-fold increase in secretion of a lysosomal marker enzyme β-hexosaminidase was seen, there was no increase in the amount of sialic acid esterase activity in the medium (data not shown). Also, sera from several patients with I-cell disease, which had elevated levels of other lysosomal enzymes, did not show detectable levels of the sialate:9-*O*-acetylesterase (data not shown). However, both of these negative results are confounded because the secreted unprocessed form of the esterase has not been shown to be enzymatically active. To ask directly if newly synthesized LSE carried the Man-6-P marker, we labeled cells for 1 h with [^{35}S]Met and chased for 3 h, conditions under which many other lysosomal enzymes are known to have acquired the Man-6-P recognition marker (47–49). The labeled cell lysate was then passed over a column of immobilized cation-independent Man-6-P receptor from bovine liver. Under these conditions, a significant proportion (~0.3%) of the total cellular radioactivity bound to the column and was specifically eluted with 5 mM mannose 6-phosphate. A portion of this eluate was immunoprecipitated with the polyclonal antibody against the LSE. Although several other labeled protein bands were seen in the total mannose 6-phosphate eluate, presumably representing other lysosomal enzymes, the LSE is not found in this fraction, indicating that it had not been retained by the Man-6-P receptor column (data not shown).

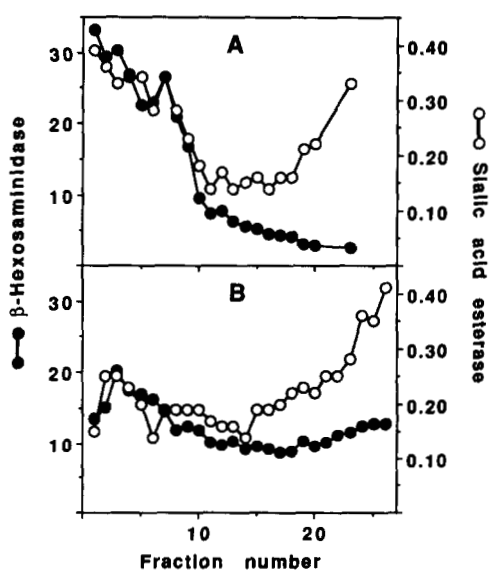

FIG. 5. **Triton TWR 1339-induced shift of the LSE and β-hexosaminidase on sucrose gradients.** Rats were injected with Triton TWR 1339 and liver homogenates fractionated on continuous sucrose gradients as described under "Experimental Procedures." β-Hexosaminidase (*closed circles*) and 9-*O*-acetylsialic acid esterase activity (*open circles*) were assayed as described under "Experimental Procedures." The esterase activity in the upper part of the gradient arises from the unrelated cytosolic enzyme. Marker enzymes for other organelles (Golgi, mitochondria, ER) did not show a significant qualitative change in distribution profile (data not shown).

DISCUSSION

The CSE and LSE of rat liver are both water-soluble proteins with similar enzymatic activities. They were initially defined as separate enzymes on the basis of their ConA binding properties (the LSE bound, whereas the CSE did not) and their apparent subcellular localization (the CSE appeared to be *cytosolic*, whereas the LSE was found within the *lumen* of membrane-bound compartments). The rat liver CSE also appears similar in description to the sialic acid esterase activity reported by Schauer and others (50–52) in buffer extracts of a variety of other tissues. The lack of cross-reactivity of the CSE with polyclonal antibodies against the LSE shown in this study indicates that it is indeed a distinct protein. This is not entirely surprising, since there is no other known precedent for a single protein that shares a localization both within the cytosol and within the lumen of the ER-Golgi-plasmalemma pathway. We have previously suggested that the primary role of the CSE is to "recycle" *O*-acetylated sialic acids, if they are delivered intact to the cytosol (53).

In our previous study, a co-purifying protein with a slightly higher molecular weight was separated from the LSE only at the final procion red-agarose purification step. This protein also turned to be an esterase, with activity against 4MU-acetate, but not against 9-*O*-acetyl sialic acids (6). The possibility remained, however, that this contaminating esterase was a partially inactive precursor form of the LSE. In the present study, we have shown by monoclonal and polyclonal antibodies and by amino-terminal sequencing that this is not the case. The contaminating esterase shows close similarity to previously described gene family of "microsomal" ER esterases of the rat liver (16–28). In contrast, the amino-terminal sequences of the LSE subunits indicate a distinct enzyme, with no sequence homology to any previously reported protein.

In this study, and in the accompanying paper (7), we have provided evidence that the LSE may be predominantly localized to lysosomes. In recent preliminary work, we have also used immuno-electron microscopy to obtain further evidence for the lysosomal and late endosomal localization of the LSE.[3] Several other features of the LSE are similar to those of other lysosomal enzymes. First, like most lysosomal hydrolases, the LSE is a water-soluble *N*-linked glycoprotein that passes through the Golgi apparatus during its biosynthesis. Second, it undergoes a late proteolytic cleavage that is reminiscent of that seen with some lysosomal enzymes such as cathepsin D (36–39). Third, a portion of the newly synthesized enzyme is secreted into the medium in an uncleaved form. On the other hand, our studies to date suggest that the newly synthesized LSE may not carry the Man-6-P recognition marker. However, since all of our data are negative, we cannot yet rule out

[3] C. Butor, G. Griffiths, N. Aronson, and A. Varki, unpublished observations.

the possibility that the enzyme carries the modification very transiently during its biosynthesis. Further studies of the LSE biosynthesis in multiple cell types and direct structural analysis of the sugar chains on the enzyme will be required to elucidate the mechanism of trafficking to the lysosome. In any event, many lysosomal enzymes are known to follow a Man-6-P receptor-independent pathway to the lysosome, in cell types such as hepatocytes (38, 42, 54–60). At least in some cases, *e.g.* cathepsin D, this may be related to an unexplained membrane association of newly synthesized enzyme (42, 61, 62). In this regard, it is noteworthy that a small but significant portion of the sialate:9-*O*-acetylesterase activity remains associated with rat liver membranes even after repeated freeze-thaw and/or saponin permeabilization.

The probable role of this esterase in the turnover of *O*-acetylated sialic acids has been discussed to some extent in the accompanying paper (7). Many microbial sialidases show diminished activity against sialic acids with *O*-acetyl esters on their side chain. At present, there is no information regarding the effect of side chain *O*-acetylation on the action of the mammalian lysosomal sialidases. Assuming that the LSE is indeed located in late endosomes and lysosomes, its primary role might be to de-*O*-acetylate sialic acids prior to the action of these lysosomal sialidases. However, it is also possible that the LSE could play a role in de-*O*-acetylating sialic acids on glycoconjugates that are destined for recycling rather than degradation. In this regard, we have reported that the *O*-acetyl esters on the ganglioside G_{D3} in human melanoma cells turn over faster than the underlying sialic acids of the molecule (63).

The LSE can act only on *O*-acetyl esters at the 9-position of sialic acids. However, we have previously shown that it is capable of de-*O*-acetylating both di-*O*-acetyl- and tri-*O*-acetyl-*N*-acetylsialic acids by first cleaving the *O*-acetyl ester at the 9-position. *O*-Acetyl esters at the 7- and 8-*O*-positions then undergo spontaneous migration to the 9-position, where they can then be cleaved (6). Of course, the rate of such migration, especially from the 7-position, is extremely pH-dependent (64, 65). It is possible that at the acidic pH usually found in lysosomes, the 7-*O*-acetyl esters remain stable, to serve some as yet unknown biological role.

Acknowledgments—We are indebted to Sandra Diaz for help with some of the experiments and for critical reading of the manuscript. We also thank Dr. R. Doolittle for help with examining the amino acid sequences for homology.

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