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SPECIAL CONSIDERATIONS IN THE PURIFICATION OF THE GM_3 GANGLIOSIDE-FORMING ENZYME, CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1): EFFECTS OF PROTEASE INHIBITORS ON RAT HEPATIC SAT-1 ACTIVITY*

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SUMMARY Co-purification of an endogenous proteolytic activity has been proposed as the cause for the size heterogeneity of sialyltransferases. Reported herein are results on the effects of various protease inhibitors, sulfhydryl-reducing agents and antimicrobial agents on SAT-1 activity. Addition of protease inhibitors to immunoaffinity-purified rat liver SAT-1 dramatically affects its activity. All protease inhibitors examined, with the exception of PMSF, inhibited the purified enzyme. The most inhibitory were the cysteine (thiol) protease inhibitors. This effect is less spectacular when the effect of these inhibitors was studied on SAT-1 activity in Golgi-enriched microsomes, although the inhibition was greatest by the cysteine protease inhibitors. One dramatic effect, found in both cases, was the apparent activation of SAT-1 activity in the presence of β-mercaptoethanol.

Intracellular proteases and hydrolases perform a variety of highly controlled, necessary biological functions, such as the regulation of cellular proliferation (for review, 1). For example, in glycosphingolipid metabolism, specific hydrolases cleave bioreactive groups from the carbohydrate or lipid moieties. The catabolites formed alter the modulation of the cell surface. For example, the cell-dependent degradation of GM_3 ganglioside by sialidase to lactosylceramide (LacCer)[2] (2-5) or by cerebrosidase to lyso-GM_3 (6) may relieve GM_3 inhibition of EGF-receptor tyrosine kinase autophosphorylation, thus allowing cell proliferation to continue (7,8). GM_3 and its catabolites also affect the protein kinase C (PK-C) signal transduction

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The abbreviations used are: APMSF, (4-amidino-phenyl)-methane-sulfonyl fluoride; β-ME, 2-mercaptoethanol; CMP, cytidine 5'-monophosphate; DTT, dithiotheitol; EGF, epidermal growth factor; GD_3, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1 Ceramide; GM_3, NeuAcα2-3Galβ1-4Glcβ1-1 Ceramide; GM_1, Galβ1-3GaNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1 Ceramide; GD_{1a}, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1 Ceramide; Glycosphingolipid (all abbreviations for Glycosphingolipids are according to the Svennerholm nomenclature (38) and the IUPAC-IUB recommendations (39)); Kd, kilodalton, LDAO, lauryl dimethylamine oxide, also known as Ammonyx LO; PK-C, protein kinase C; PMSF, phenylmethane-sulfonyl fluoride; SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase; SAT-2, CMP-sialic acid:GM_3 α2-8 sialyltransferase, GD_3 synthase; SAT-4, CMP-sialic acid:GM_1 α2-3 sialyltransferase, GD_{1a} synthase; SDS-PAGE, sodium dodecyl sulfate polyacrylaminde gel electrophoresis; ST, sialyltransferase; TLCK-HCl, (L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl; TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone.

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mechanism in a manner antagonistic to that observed for EGF-receptor (for review, 9). Catabolites of GM_3 ganglioside are postulated to be internalized and recycled back to the endoplasmic reticulum and Golgi for resynthesis of GM_3 (4,10).

Another function for proteolytic processing in complex carbohydrate metabolism has been proposed as a mechanism for the release of soluble, active forms of glycosyltransferases to the extracellular matrix through the site-specific cleavage of these enzymes within the "stem" region (11,12). Glycosyltransferases are the enzymes responsible for extending the carbohydrate chains of glycoproteins and glycolipids. The presence of endogenous proteases within the Golgi, responsible for these cleavages, complicate purification of intact glycosyltransferases from the Golgi. We report here our observations, made during the purification of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1), on the effects of various protease inhibitors on SAT-1 activity.

MATERIALS AND METHODS

Materials - Cytidine 5'-monophosphate sialic acid, (CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid, 286.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and its specific activity adjusted to 22,200 dpm/nmol with unlabeled CMP-sialic acid from Sigma (St. Louis, MO). The sphinganine-containing form of lactosylceramide was purchased from Sigma and used in activity assays. Ammonyx LO (lauryldimethylamine oxide, LDAO) was obtained from the Stepan Company (Northfield, IL). EDTA, azide and sodium cacodylate were purchased from Sigma Chemical Company (St. Louis, MO). PMSF, APMSF, Leupeptin, aprotinin, pepstatin, TPCK, TLCK-HCl, α_2-macroglobulin (and α_2-macroglobulin carrier-fixed), E-64 and dithiothreitol (DTT) were purchased from Boehringer Mannheim (Indianapolis, IN). Ep-459 and Ep-475 were gifts from Dr. Hanada of Taisho Pharmaceutical (Tokyo, Japan). Ultrapure electrophoretic-grade β-mercaptoethanol was from Bio Rad (Richmond, CA). Sep Pak C_{18} cartridges were purchased from Waters Associates (Milford, MA). All other reagents were ultrapure or ACS grade from commercial sources.

Preparation of Golgi-enriched Microsomes. Golgi vesicles were isolated from rat liver by well-established procedures (13-17) as described in detail (18) in 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose in the absence of protease inhibitors.

Purification of CMP-sialic acid:lactosylceramide α2-3 Sialyltransferase. CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) was purified from rat liver Golgi by immunoaffinity chromatography using M12GC7 anti-SAT-1 monoclonal antibody coupled to Affi Gel 10 as previously described for the purification of SAT-1 from detergent-extracted rat liver Golgi vesicles (18).

CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) Activity Assay. SAT-1 assays, with radiolabeled CMP-sialic acid as the donor substrate and lactosylceramide as the acceptor substrate, were carried out by adsorbing 5 nmol LacCer to the microtiter plates in 50% ethanol as described (19). The plates were washed with PBS and blocked with 5% (w/v) bovine serum albumin (BSA) for 30 min. After rinsing, 20 nmol of CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid (New England Nuclear, lot # 2655-018, specific activity adjusted to 20,000 dpm/nmol) was added. To the reaction wells were added sonicated mixtures containing the following components: 25 µl of assay buffer (200 mM sodium cacodylate (pH 6.5), 20 mM $MnCl_2$ and 0.3% (v/v) lauryldimethylamine oxide (LDAO) and 25 µl of SAT-1 enzyme or Golgi (0.005-0.1 mg protein). The plate was covered and incubated in a humidified environment at 37°C for 2 hr. Following the incubation period, the reaction mixture was removed and the microtiter wells washed 3 - 5 times with PBS. The wells were put into 5 ml Safety-Solve liquid scintillant and the amount of [^{14}C]-GM_3 formed counted in a Packard (model #460C) liquid scintillation counter.

RESULTS AND DISCUSSION

The homogeneity of the affinity-purified SAT-1 was extremely difficult to verify because of the size heterogeneity of the enzyme in electrophoretic patterns (18). The pattern generated could be varied depending on the temperature and time of solubilization in SDS-PAGE sample buffer.

TABLE 1

SIZE HETEROGENEITY OF SIALYLTRANSFERASE

Reference	Specificity	Source	Fold Purification	Molecular weights (Kd)
Paulson *et al.* (1977) JBC **252**, 2356.	Galβ1-4GlcNAc α2-3ST	Bovine Colostrum	440,000-X	56[1] 43
Sadler *et al.* (1979) JBC **254**, 4434.	Gal α2-3ST	Porcine Submaxillary Gland	92,200-X	49[1] 44
Sadler *et al.* (1979) JBC **254**, 5934.	GalNAc α2-6ST	Porcine Submaxillary Gland	117,000-X	172 160 100 80 69 56
Weinstein *et al.* (1982) JBC **257**, 13835.	Galβ1-4GlcNAc α2-6ST	Rat Liver	23,000-X	47[1] 43
Weinstein *et al.* (1982) JBC **257**, 13835.	Galβ1-3(4)GlcNAc α2-3ST	Rat Liver	860,000-X	56[1] 44
Joziasse *et al.* (1985) JBC **260**, 4941.	Galβ1-3GlcNAc α2-3ST and α2-3SAT (SAT-4)	Human Placenta	20,000-X	65[1] 43 41 40
Gu *et al.* (1990) BBRC **166**, 387-393.	NeuAcα2-3Galβ1-4Glcβ1-1Cer α2-8SAT (SAT-2)	Rat Liver	10,000-X	63[2] 59[2] 55[1] 51[2] 43[2]
Melkerson-Watson and Sweeley (1991) JBC, in press	Galβ1-4Glcβ1-1Cer α2-3SAT (SAT-1)	Rat Liver	42,800-X	60[1] 56 50 47

[1] Apparent molecular weights of the purified sialyltransferases.

[2] Molecular weight values were estimated form the SDS-PAGE pattern in Figure 3 of Gu *et al.* (25).

While there are several explanations for these data, similar size heterogeneity has been observed with other purified sialyltransferases (Table 1). Proteolytic degradation and mechanical disruption of the enzymes from the Golgi during their purifications were considered as possible causes for the heterogeneity of these purified enzymes. Recently, this concept of proteolytic processing was addressed in a review by Paulson and Colley (12). The cDNA's from four known glycosyltransferases exhibit some homology with regard to gross structure. They all possess a cytosolic NH_2 terminus, a hydrophobic transmembrane domain, a "stem" region, and a Golgi lumenal COOH terminus. The "stem" region between the transmembrane and catalytic domain has been implicated as the necessary element in the anchoring and targeting of the glycosyltransferase in the protein transport between the endoplasmic reticulum and the Golgi. It is also the apparent site of proteolysis (26). The endogenous proteolytic activity, suggested to be a cathepsin D-like activity (27), releases the catalytically active C-terminus of Galβ1-4GlcNAc α2-6

TABLE 2

EFFECTS OF PROTEASE INHIBITORS ON IMMUNOAFFINITY-PURIFIED SAT-1 ACTIVITY

Inhibitor	Inhibitor Specificity	Concentration	dpm [^{14}C]-GM_3	% of Control
Control	none	0.0	850 30	100%
PMSF	serine proteases	1000 μM	1120	132%
APMSF	serine proteases	20 μM	440	52%
Aprotinin	serine proteases	0.3 μM	220	26%
Leupeptin	serine and thiol proteases (e.g., cathepsin B & L)	1 μM	240	28%
TLCK-HCl	trypsin & thiol proteases	135 μM	230	27%
TPCK	chymotrypsin & thiol proteases	284 μM	280	33%
E-64	thiol proteases (e.g., cathepsin B)	2.8 μM	250	29%
Ep-459	thiol proteases (e.g., cathepsin D)	2.8 μM	210	25%
Ep-475	thiol proteases	2.8 μM	140	16%
Pepstatin A	acid proteases (e.g., cathepsin D)	1 μM	240	28%
α_2-Macroglobulin	general endoproteases	1 unit	280	33%
EDTA	metalloproteases	100 μM	250	29%
DTT	sulfhydryl reducing agent	100 μM	90	11%
β-ME	sulfhydryl reducing agent	180 μM	3330	392%
Azide	antimicrobrial agent	1000 μM	110	13%

sialyltransferase (CMP-N-acetylneuraminate:β-galactoside α2-6 sialyltransferase, EC 2.4.99.1) from the lumenal face of the *trans*-Golgi for transport out of the cell during acute-phase response.

Therefore, to minimize proteolytic degradation during the purification of SAT-1, anti-SAT-1 specific monoclonal antibody, M12GC7, (18) was used to immunoaffinity-purify the enzyme from lauryldimethylamine oxide-extracted Golgi membrane proteins (28) in the presence of several protease inhibitors including leupeptin, pepstatin and E-64 (and its analogs Ep-459 and Ep-475, gifts from Dr. Hanada of Taisho Pharmaceuticals, Japan (29)), all of which are potent cathepsin inhibitors. The enzyme was purified to homogeneity with an apparent molecular weight of 60,000. The enzyme was inactivated, but was immunologically reactive on Western blots with M12GC7. We speculated that this inactivation was due to one or more of the cysteine (thiol) protease inhibitors (TLCK-HCl, TPCK, E-64, or Ep-459), which is consistent with the finding that a thiol (R-SH) group has been found in the region of the CMP-NeuNAc binding site of β-galactoside α2-6 sialyltransferase (30).

A summary of the effects of these and other protease inhibitors, as well as some sulfhydryl reducing and antibacterial agents used in SDS-PAGE and protein chromatography column preservation, are listed in Table 2 along with their effect on SAT-1 activity. SAT-1 was immunoaffinity-purified from LDAO-extracted rat liver Golgi-enriched fraction as previously described elsewhere (18). No protease inhibitors were added to the purification buffer. Various protease inhibitors, at the concentrations specified, were added to immunoaffinity-purified SAT-1 and assayed for activity (Table 2). Of the inhibitors tested,

only the serine protease inhibitor PMSF, common in most glycosyltransferase purifications (18,20-25), did not inhibit the enzyme. All other protease inhibitors significantly inactivated the purified SAT-1 48-87% under the conditions employed. The most inhibitory substances were the cysteine (thiol) protease inhibitors (leupeptin, TLCK-HCl, TPCK, E-64, Ep-459, and Ep-475), suggesting that SAT-1 may also contain a thiol group in or near its sugar nucleotide-binding site, as had been reported for β-galactoside α2-6 sialyltransferase (30). Some sequence homology has been reported for the sugar-nucleotide binding region in other glycosyltransferases (for review, 12).

Pepstatin, a potent inhibitor of cathepsin D, also inhibited SAT-1. Cathepsin D, is a thiol protease with a heavy metal requirement, exhibits a preference for peptides flanked by hydrophobic amino acid residues (1). Addition of EDTA can inhibit cathepsin D proteolytic degradation, but EDTA also inhibits SAT-1 activity, since SAT-1 has a divalent cation requirement for activity (18,31). A 10,000-fold purification of a related glycolipid sialyltransferase, GD_3 synthase (NeuAcα2-3Galβ1-4Glcβ1-1Ceramide α2-8 sialyltransferase, SAT-2), has recently been reported (25); this purification was carried out in the presence of 1 mM EDTA and 10 mM β-mercaptoethanol (ß-ME). Analysis of SAT-2 by SDS-PAGE showed size heterogeneity. The predominant molecular weight was reported to be 55,000 daltons (25). The combination of EDTA and β-ME in their purification buffer apparently did not inhibit proteolysis.

Our investigation of the effects of sulfhydryl reducing agents, β-ME and dithiothreitol (DTT), commonly used in SDS-PAGE sample buffer, indicates that the addition of β-ME at a final concentration of 0.18 mM enhances SAT-1 activity about 4-fold relative to control. Thus, there is potential use of β-ME in concert with the appropriate concentration of protease inhibitors (e.g., EDTA or pepsatin) for the inhibition of cathepsin D proteolytic degradation of sialyltransferases during purification while maintaining active fractions for monitoring purification of these enzymes.

In a previous study, summarized in Table 3, are the results of adding the same concentrations of protease inhibitors to Golgi-enriched microsomal fraction. The inhibition of SAT-1 activity was not as dramatic. SAT-1 activity was stable in microsomes treated with 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 1 unit α_2-macroglobulin, and 2.8 μM E-64. The cysteine (thiol proteinase inhibitors, TLCK-HCl, TPCK, Ep-459 and Ep-475 inhibited SAT-1 20-40%. EDTA (100 μM) inhibited SAT-1. The greatest inhibition of SAT-1 activity when assayed in Golgi-enriched microsomes was observed at 63% of control following the addition of either Ep-459 or EDTA. Addition of 0.1 mM β-ME resulted in an 11-fold enhancement of SAT-1 activity in intact Golgi.

Not only should the effects of protease inhibitors on the inhibition of proteolytic degradation and enzyme activity be considered, but so should the differential effects of detergents on both proteinase activity(ies) during purification as well as the detergent effects on the glycosyltransferase being purified. We recently reported the effects of lauryldimethylamine oxide (LDAO) on SAT-1 (28). SAT-1 activity and stability are dependent upon the concentration of LDAO used. Detergents have also been shown to stabilize and activate proteinase activity. Arribas and Castano (32) found that low concentrations of detergents (0.01%) such as Triton X-100, SDS and CHAPS activate the hydrolysis of protein substrates by proteases, while higher concentrations (0.1%) inhibit degradation. A similar argument may apply to LDAO. LDAO may stabilize the sialyltransferase activity, and, in addition, stabilize (and potentially activate) the endogenous proteolytic activity associated with the purification of these enzymes. Another possibility is that

TABLE 3

EFFECTS OF PROTEASE INHIBITORS ON SAT-1 ACTIVITY IN GOLGI-ENRICHED MICROSOMES

Inhibitor	Inhibitor Specificity	Concentration	dpm [^{14}C]-GM_3	% of Control
Control	none	0.0	104 18	100%
PMSF	serine proteases	1000 μM	108 19	104%
APMSF	serine proteases	20 μM	86 11	83%
Aprotinin	serine proteases	0.3 μM	71 12	68%
Leupeptin	serine and thiol proteases (e.g., cathepsin B & L)	1 μM	220 36	212%
TLCK-HCl	trypsin & thiol proteases	135 μM	76 17	73%
TPCK	chymotrypsin & thiol proteases	284 μM	82 18	79%
E-64	thiol proteases (e.g., cathepsin B)	2.8 μM	155 87	149%
Ep-459	thiol proteases (e.g., cathepsin D)	2.8 μM	65 13	63%
Ep-475	thiol proteases	2.8 μM	90 13	86%
Pepstatin A	acid proteases (e.g., cathepsin D)	1 μM	154 63	148%
α_2-Macroglobulin	general endoproteases	1 unit	112 42	108%
EDTA	metalloproteases	100 μM	66 6	63%
DTT	sulfhydryl reducing agent	100 μM	66 6	63%
β-ME	sulfhydryl reducing agent	180 μM	1170 155	1120%

the associated endogenous protease is a lysosomal proteinase (like cathepsin D) which co-purifies with SAT-1 in Golgi-enriched microsomes through the disruption and mixing of the Golgi vesicles with light lysosomes during sonication and detergent extraction. Dawson and coworkers (33) have studied the subcellular distribution of cathepsin D, as well as its pre-pro and pro forms, in human fibroblasts using Percoll density gradients. Subcellular fractionation of cathepsin D and its intermediates were found uniformly distributed throughout the gradient with a slight enrichment of the mature active form in more buoyant fractions. Further, treatment of pre-pro-forms of Cathepsin D with cysteine (thiol) proteinase inhibitors, Ep-459 or leupeptin, caused inhibition of cathepsin D processing. Treatment with Ep-475 had no effect.

Another important effect on proteinase activity in relation to SAT-1 and its purification is the stimulation of some proteases by ATP (for review, 1). Two classes of "ATP-dependent" proteases have been characterized; one requires ATP for stabilization, the second requires ATP for hydrolysis. ATP activation of cathepsins D and L has been reported (34,35). One mechanism reported for cathepsin L requires binding of ATP to the protein substrate, which increases susceptibility of the protein to proteolytic degradation (36).

Activation of SAT-1 through the phosphorylation of tyrosine residue(s) has been proposed as a mechanism of regulation of cell growth by the increase of cell surface GM_3 ganglioside during G_1 of the cell cycle (5). ATP has been demonstrated to enter the Golgi (37). Potentially, ATP-stabilization of protease activity or ATP-dependent hydrolysis of enzyme by proteases may play a role in the mechanism for the proteolytic degradation associated with SAT-1 sialyltransferase during its purification.

CONCLUSIONS

From these observations and considerations, SAT-1 purifications are now carried out under the following conditions to inhibit proteolytic degradation during its purification. SAT-1 is typically immunoaffinity purified from 20 g (wet weight) rat liver which has been perfused with 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose, 1 mM PMSF, 1 μM leupeptin and 1 μM pepstatin A. Following homogenization in five volumes of the same buffer, the nuclei, mitochondria and cellular debris are removed by centrifugation at 5000 x g for 10 min. The Golgi-enriched microsomes are collected by centrifugation at 100,000 x g and detergent-extracted with LDAO as previously described (18). The LDAO-soluble proteins are reacted (batch method) with 2 ml carrier-fixed α_2-macroglobulin for 30 min and proteases liberated during detergent extraction are coupled to carrier-fixed α_2-macroglobulin which is removed following centrifugation (as described by the manufacturer, BMB).

The relationship between the effects of protease inhibitors on the size heterogeneity of SAT-1 and on SAT-1 glycosyltransferase activity is unknown. The data are consistent, to a first approximation, with the hypothesis that proteolytic cleavage of SAT-1 during purification is primarily due to the action of a cathepsin-like thiol protease. Inhibition of SAT-1 glycosyltransferase activity by protease inhibitors, and activation by β-mercaptoethanol, may occur by modification of thiol residues necessary for SAT-1 activity. Thus, attempts to inhibit protease activity generally lead to coincident inhibition of SAT-1 catalytic activity. The inhibition of SAT-1 catalytic activity by α_2-macroglobulin, however, does not fit this pattern, and remains to be explained.

The correlation between inhibition of SAT-1 proteolysis and catalysis may also imply a physiologically significant interaction between SAT-1 and a protease activity for which several speculative mechanisms can be envisioned: (a) a specific protease activity may be required for SAT-1 activity, and may be involved in the regulation of SAT-1 acivity during the cell cycle, (b) an endogenous protease may form part of a complex with SAT-1, or (c) SAT-1 may possess endogenous protease activity.

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