The role of a cathepsin D-like activity in the release of Gal β 1-4GlcNAc α 2-6-sialyltransferase from rat liver Golgi membranes during the acute-phase response

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Golgi-membrane-bound Gal β 1-4GlcNAc α 2-6-sialyltransferase (CMP-*N*-acetylneuraminic acid: β -galactoside α 2-6-sialyltransferase, EC 2.4.99.1) behaves as an acute-phase reactant increasing about 5-fold in serum in rats suffering from inflammation [1]. The mechanism of release from the Golgi membrane is not understood. In the present study it was found that sialyltransferase could be released from the membrane by treatment with ultrasonic vibration (sonication) followed by incubation at reduced pH. Maximum release occurred at pH 5.6, and membranes from inflamed rats released more enzyme than did membranes from controls. Galactosyltransferase (UDP-galactose: *N*-acetylglucosamine galactosyltransferase; EC 2.4.1.38), another Golgi-located enzyme, which does not behave as an acute-phase reactant, remained bound to the membranes under the same conditions. Release of the α 2-6-sialyltransferase from Golgi membranes was substantially inhibited by pepstatin A, a potent inhibitor of cathepsin D-like proteinases. Inhibition of release of the sialyltransferase also occurred after preincubation of sonicated Golgi membranes with antiserum raised against rat liver lysosomal cathepsin D. Addition of bovine spleen cathepsin D to incubation mixtures of sonicated Golgi membranes caused enhanced release of the sialyltransferase. Intact Golgi membranes were incubated at lowered pH in presence of pepstatin A to inhibit any proteinase activity at the cytosolic face; subsequent sonication showed that the sialyltransferase had been released, suggesting that the proteinase was active at the luminal face of the Golgi. Golgi membranes contained a low level of cathepsin D activity (EC 3.4.23.5); the enzyme was mainly membrane-bound, since it could only be released by extraction with Triton X-100 or incubation of sonicated Golgi membranes with 5 mM-mannose 6-phosphate. Immunoblot analysis showed that the transferase released from sonicated Golgi membranes at lowered pH had an apparent M_r of about 42000 compared with one of about 49000 for the membrane-bound enzyme. Values of K_m for the bound and released enzyme activities were comparable and were similar to values reported previously for liver and serum enzymes. The work suggests that a major portion of sialyltransferase containing the catalytic site is released from a membrane anchor by a cathepsin D-like proteinase located at the luminal face of the Golgi and that this explains the acute-phase behaviour of this enzyme.

INTRODUCTION

Inflammation in humans and experimental animals caused by chemical inflammatory agents, bacterial infections, neoplastic disease and a variety of other conditions results in significant biochemical and physiological changes known as the acute-phase response [1–5]. A major change is an increase in the total protein-bound carbohydrate of serum arising from an increase in the circulating levels of a variety of serum glycoproteins. Glycoproteins that increase in this way are referred to as acute-phase reactants [2]. The acute-phase reactants are mainly liver-synthesized secreted glycoproteins such as α_1 -acid glycoprotein, fibrinogen and haptoglobin [3,4]. In recent work we identified Gal β 1-4GlcNAc α 2-6-sialyltransferase (CMP-*N*-acetylneuraminic acid: β -galactoside α 2-6-sialyltransferase, EC 2.4.99.1) as an acute-phase reactant [1,6,7]. This enzyme is responsible for attaching NeuAc in an α 2-6 linkage to the terminal positions of *N*-linked chains of glycoproteins. The enzyme is membrane-bound and is located on the luminal

face of the *trans*-Golgi compartment of the hepatocyte [8]. It is elevated about 2-fold in the Golgi membranes during the acute-phase response [1], and this is accompanied by a substantial release of enzyme activity from the membranes resulting in a 5-fold increase in enzyme activity in the circulation compared with normal animals [1,6]. Galactosyltransferase (EC 2.4.1.38), another *trans*-Golgi-located enzyme, is also elevated about 2-fold in liver under the same conditions, but this enzyme is not released from liver in inflammation, so it does not behave as an acute-phase reactant [1]. The sialyltransferase thus represents a new type of acute-phase reactant in which an enzyme which is normally membrane-bound is released in large amounts from the liver, resulting in substantial increases in extracellular concentrations.

As part of our studies on the acute-phase response of sialyltransferase we have now examined the mechanism of release of the enzyme from the Golgi membranes. Here we show that Gal β 1-4GlcNAc α 2-6-sialyltransferase is particularly sensitive to the action of an endogenous proteinase, which cleaves a portion of the

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetylglucosamine; Gal, galactose; CMP-NeuAc, CMP-*N*-acetylneuraminic acid; UDP-Gal, UDP-galactose.

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enzyme containing the catalytic site from a membrane anchor and this allows the enzyme to be released into the extracellular space; galactosyltransferase is not released under the same conditions. Evidence is presented to show that the proteinase that releases sialyltransferase from the Golgi membrane has the properties of the aspartic proteinase cathepsin D (EC 3.4.23.5)[9], and the proteinase is active in releasing sialyltransferase at the luminal side of the Golgi membrane.

MATERIALS AND METHODS

Materials

CMP-[4,5,6,7,8,9- ^{14}C]NeuAc (247 mCi/mmol) and UDP-[U- ^{14}C]Gal (340 mCi/mmol) were obtained from New England Nuclear Corp., Lachine, Quebec, Canada; CMP-NeuAc, UDP-Gal, CDP-choline, penicillin-G (sodium salt), streptomycin, soybean trypsin inhibitor, antipain, aprotinin, bestatin, α_1 -antitrypsin, jack-bean-meal β -galactosidase, cathepsin D (bovine spleen), Triton X-100 and mannose 6-phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Leupeptin, and α_2 -macroglobulin were obtained from Boehringer Mannheim, Dorval, Quebec, Canada; pepstatin A was from Sigma or Boehringer Mannheim. Rat α_1 -acid glycoprotein was prepared as previously described [10,11]. Rat asialo- α_1 -acid glycoprotein and asialoagalacto- α_1 -acid glycoprotein were prepared as previously described [1]. All other chemicals were of analytical-reagent grade and were of commercial origin.

Preparation of Golgi membranes

Inflammation was induced in 150–200 g male Long-Evans hooded rats by subcutaneous injection of 0.5 ml of oil of turpentine per 100 g body weight as described previously [11]; controls received injections of sterile 0.15 M-NaCl. Animals were killed 36 h after injection by severing the jugular veins as described previously [10,11]. Livers were perfused *in situ* via the portal vein with ice-cold 0.15 M-NaCl. Golgi membranes were prepared as described by Leelavathi [12] and as modified by Moreman & Touster [13]; Golgi membranes prepared in this way are free of lysosomal material [12,13]. All sucrose solutions were made up in 0.1 M-potassium phosphate buffer (pH 6.5)/5 mM-MgCl₂. Excised livers were minced by hand and homogenized in 4 vol. of 0.5 M-sucrose using four up-and-down strokes of a Potter-Elvehjem homogenizer over 30 s at 1000 rev./min. A Beckman L5-70 ultracentrifuge was used throughout. The liver homogenates were centrifuged for 10 min at 600 g in the Ti 60 rotor to sediment large-granule material. The resultant supernatants were layered on top of 20 ml of 1.3 M-sucrose solution and centrifuged for 90 min at 75000 g in a Ti 60 rotor. The Golgi band appearing just above the 1.3 M-sucrose layer was aspirated, adjusted to 1.1 M-sucrose and 10 ml samples were layered on top of 20 ml of 1.25 M-sucrose solution, covered with a 0.5 M-sucrose solution, and centrifuged for 90 min at 80000 g in the SW 28 rotor. The dense white Golgi bands appearing between the 0.5 M and 1.1 M layers were removed by aspiration and subjected once more to an identical discontinuous sucrose-density-gradient centrifugation. The resultant Golgi bands were pooled, adjusted to 0.25 M-sucrose, and pelleted by centrifuging at 175000 g in the Ti 60 rotor for 45 min. Golgi membranes prepared in this way typically exhibited 40-fold increases in specific

enzyme activities of sialyltransferase compared with the homogenates from which they were derived.

Golgi pellets were resuspended in 0.25 M-sucrose and ultrasonically vibrated (sonicated), in 2.5 ml portions, at 50 W utilizing a 1 mm-diameter titanium probe (Artek dismembrator). Sonicated and non-sonicated Golgi-membrane suspensions were adjusted to various pH values (see the Results section) with citrate buffers [14], keeping the molarity constant at 50 mM. Protein concentration was kept at 1 mg of Golgi membrane protein/ml, unless otherwise stated, and the incubation volume was typically 0.5 ml. When proteinase inhibitors were added, concentrations of up to 10^{-3} M were used for leupeptin, antipain, aprotinin and bestatin; soybean trypsin inhibitor, α_1 -antitrypsin and α_2 -macroglobulin were tested at concentrations up to 100 $\mu\text{g}/\text{ml}$; pepstatin A was used at 10^{-3} – 10^{-11} M and was added as a stock solution in dimethyl sulphoxide. Samples were incubated for up to 30 min at 37 °C, cooled in ice and adjusted to pH 7.0 with 0.1 M-imidazole/HCl buffer, pH 9.0, and the final volumes adjusted to 1.0 ml with water; samples were centrifuged at 175000 g in the Ti 70 rotor for 45 min to sediment particulate material. Samples (0.5 ml) of the supernatant were carefully removed from the tubes, and the pellets were resuspended in 0.25 M-sucrose by gentle homogenization using a 1 ml-capacity Potter-Elvehjem homogenizer; samples were assayed immediately for sialyltransferase and galactosyltransferase activities (see below). When bovine spleen cathepsin D was added to Golgi suspensions it was present at 1 $\mu\text{g}/\text{ml}$ and incubation was for up to 12 min. In experiments where antiserum containing antibodies raised against rat liver lysosomal cathepsin D were used, incubations were scaled down to 0.1 ml of Golgi suspension (300 μg of Golgi protein); up to 29 μl of antiserum were added to Golgi suspensions immediately after sonication, and samples were incubated at pH 7.0 for 45 min at 37 °C; after incubation, pH values were adjusted to 5.6 and incubation was continued as described above. At the end of incubation, pH was adjusted to 7.0, samples were centrifuged and supernatants were assayed for sialyltransferase. Antiserum raised against rat liver lysosomal cathepsin D was kindly given by Dr. B. Wiederanders (Martin Luther University, German Democratic Republic). Control experiments showed that treatment of Golgi membranes with ultrasonic vibration as described above had no effect on sialyltransferase or galactosyltransferase activities.

Experiments were also performed to determine if the proteinase that was responsible for release of sialyltransferase was located on the luminal face of the Golgi and was more effective in releasing sialyltransferase with Golgi material prepared from inflamed animals. In these experiments Golgi membranes were isolated as described above and incubated for up to 30 min at pH 5.6, but without prior sonication. Pepstatin A at a concentration of 10^{-6} M was present in the incubation mixture to inhibit any residual proteinase that might have been released from the Golgi during homogenization and resuspension. After incubation, a 400 μl sample was removed, centrifuged and the supernatant assayed for sialyltransferase. The remaining sample was sonicated, centrifuged, and the supernatant assayed for sialyltransferase. Sialyltransferase activity showing up in the second supernatant is likely to have been released from the luminal side of closed vesicles.

In some experiments antiserum containing antibodies against rat hepatic Gal β 1-4GlcNAc α 2-6-sialyltransferase was used to characterize the enzyme released from Golgi membranes [6,7]. In these experiments, up to 100 nl of antiserum were added to supernatant samples and incubated at pH 7.0 for 45 min at 37 °C before assay of sialyltransferase, suitable controls contained appropriate volumes of normal rabbit serum. Antiserum containing antibodies raised against sialyltransferase was kindly given by Dr. J. C. Paulson (University of California, Los Angeles, CA, U.S.A.)

Enzyme assays

Sialyltransferase and galactosyltransferase activities were assayed in liver homogenate, Golgi protein and supernatant fractions by using procedures described previously [1,3,4]. For sialyltransferase assay the incubation mixture contained 250 μ g of rat asialo- α ₁-acid glycoprotein as acceptor, 7.5 μ mol of imidazole/HCl buffer, pH 7.0 and 5 nmol of CMP-[¹⁴C]NeuAc (20 nCi/nmol); enzyme consisted of up to 110 μ l of Golgi membrane supernatant, resuspended Golgi pellet or liver homogenate; 400 nmol of CDP-choline was present in all assays to inhibit non-specific pyrophosphatase activity as described previously [1], and Triton X-100 at 1 % final concentration was present in all assays containing particulate material. The final assay volume was 0.15 ml. The assay system for galactosyltransferase was similar to that for sialyltransferase, except that the nucleotide-sugar donor was UDP-[¹⁴C]Gal (20 nCi/nmol) and the acceptor was rat asialoagalacto- α ₁-acid glycoprotein; assays contained 2 μ mol of MnCl₂. Incubations for sialyl- and galactosyl-transferases were for up to 90 min at 37 °C and were stopped by transferring to ice. Samples (60 μ l) were spotted on 2.5 cm-diameter circles of Whatman no. 1 filter paper, these were then rapidly transferred to ice-cold 10 % (w/v) trichloroacetic acid. Circles were washed as described previously [1,3,4] and counted for radioactivity in a liquid-scintillation spectrometer with 10 ml of ACS scintillant mixture (Amersham Corp.). Values for K_m were determined with asialo- α ₁-acid glycoprotein as substrate, as previously described [6]. In all enzyme assays conditions were such that product formation was linear with time and the amount of enzyme protein used. Endogenous activities were determined by using controls where exogenous acceptor was omitted; these values were subtracted from exogenous values as described previously [3]. One unit of glycosyltransferase activity is defined as equal to the transfer of 1 pmol of either NeuAc or Gal from nucleotide donor to acceptor protein/h.

For assay of cathepsin D, Golgi membranes were suspended in 0.25 M-sucrose containing 0.1 M-potassium phosphate buffer, pH 6.5, 5 mM-MgCl₂ at 10 mg of protein/ml. Cathepsin D was assayed in intact Golgi membranes and in supernatants after treatment with ultrasonic vibrations for 30 s and sedimented as described above; unsonicated controls were set up in parallel. Golgi membranes were also extracted with 0.1 % Triton X-100, or sonicated and incubated for 30 min with 5 mM-mannose 6-phosphate in 0.1 M-potassium phosphate buffer (pH 6.5)/5 mM MgCl₂; supernatants were isolated after centrifugation as described above. The assay for cathepsin D was based on the method of Anson [15] as modified by others for the use of pepstatin A [16,17]; assays were carried out in duplicate for each sample.

Assays contained up to 50 μ l of Golgi membrane suspension or supernatant fractions as appropriate in a final volume of 0.32 ml containing 0.15 M-sodium formate buffer, pH 3.3, and 3.0 % bovine haemoglobin prepared as described by Barrett [18]; 0.1 % Triton X-100 was present with intact Golgi suspensions. Samples were incubated at 37 °C for 20 min, and the reaction was stopped by the addition of 0.9 ml of 3 % (w/v) trichloroacetic acid. Precipitated protein was sedimented in an Eppendorf centrifuge and the amount of substrate degraded by proteolysis was determined from the absorbance of the supernatant at 280 nm. Proteolysis due to cathepsin D activity was calculated by subtracting the A_{280} in the presence of 5 μ g of pepstatin A/ml from that in the absence of the inhibitor (ΔA_{280}) [16–18]. A ΔA_{280} of 0.10 results from the activity of 0.10 unit of cathepsin D for 20 min incubation. All quantitative determinations were carried out in the linear range of the assay up to a net absorbance of 0.3 [15–17]. Protein was determined by the method of Lowry *et al.* [19], as modified by Miller [20].

Immunoblotting of sialyltransferase

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli [21], a Bio-Rad Mini-Protean II electrophoresis system being used. Golgi membrane and supernatant fractions were prepared for electrophoresis by heating in a boiling-water bath for 4 min in 0.125 M-Tris/HCl, pH 6.8, containing 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β -mercaptoethanol and 0.02 % (w/v) Bromophenol Blue. Samples were centrifuged for 10 min at 7000 rev./min in an Eppendorf centrifuge, and 2–10 μ l of each sample were applied to individual tracks of 10 % (w/v) acrylamide gels. Electrophoresis was for up to 3 h at 100–150 V using a tank buffer of 0.025 M-Tris/0.192 M-glycine, pH 8.3, containing 0.1 % SDS. Electroblothing was performed using the Bio-Rad Mini Trans-blot cell. Protein transfer to 0.45 μ m-pore-size nitrocellulose was for 4 h at 70V with a 0.025 M-Tris/0.192 M-glycine/20 % methanol blotting buffer, pH 8.3. Protein M_r standards were obtained from Bio-Rad and were soybean trypsin inhibitor (M_r 21 600), carbonic anhydrase (M_r 31 000), ovalbumin (M_r 42 699), bovine serum albumin (M_r 66 200) and phosphorylase *b* (M_r 97 400). Immunodetection of sialyltransferase was as described by Haas & Bright [22] using anti-sialyltransferase (1:200) and affinity-purified ¹²⁵I-protein A (Amersham Corp.); the nitrocellulose was washed, dried and autoradiographed on Hyperfilm- β max film (Amersham Corp.) for 3–7 days.

RESULTS

Effect of pH on release of sialyltransferase from Golgi membranes

Sialyltransferase is present on the luminal face of the Golgi membranes and can be released by treatment with detergents [23,24]. In the present work, mild ultrasonic treatment was used to disrupt the Golgi to expose the sialyltransferase. Experiments were carried out with Golgi membranes from control rat livers and from livers from rats suffering from inflammation for 36 h, which contain elevated sialyltransferase activities [1]. Galactosyltransferase is also located in the Golgi complex in a compartment which may not be identical with that containing sialyltransferase, but which is topologically

closely related to it [25]. Although galactosyltransferase is also elevated in liver during experimental inflammation, the enzyme is not released from liver in large quantities [1]. Release of galactosyltransferase was therefore used as an internal control in experiments designed to study the specificity of release of sialyltransferase from Golgi membranes. Fig. 1 shows that sialyltransferase was released from sonicated Golgi membranes in substantial

amounts when the pH of the incubation medium was lowered, with greater amounts of activity being released from Golgi membranes obtained from inflamed rat livers. Fig. 2 shows that 30 min incubation at pH 5.6 was sufficient to release most of the sialyltransferase; under these conditions about 70 % of the membrane-bound activity was typically released. There was negligible release of galactosyltransferase from Golgi membranes under the conditions used for the release of sialyltransferase; in all the experiments described in Figs. 1–2 galactosyl-

Fig. 1. Effect of pH on the release of sialyltransferase and galactosyltransferase from sonicated Golgi membranes

Golgi membranes were sonicated for 30 s and incubated at different pH values for 30 min. Particulate material was sedimented, and sialyltransferase and galactosyltransferase activities were determined in the supernatants. ○, ●, Sialyltransferase activity released from membranes from control (○) and 36 h-inflamed (●) rat liver Golgi membranes; △, ▲, galactosyltransferase activity released from control (△) and 36 h-inflamed (▲) rat liver Golgi membranes. The results were determined as units of glycosyltransferase activity released per mg of Golgi membrane protein and are expressed on the basis that maximum release of enzyme activity was taken as 100 %; this value represents 3117 units of sialyltransferase activity/mg of Golgi protein. This accounted for about 70 % of the total activity associated with the Golgi membranes. Each point represents the mean result from four experiments; reproducibility was within ± 10 %.

Fig. 2. Effect of incubation time on the release of sialyltransferase from sonicated Golgi membranes at pH 5.6

Golgi membranes were sonicated, then incubated at pH 5.6 for different periods of time; sialyltransferase was assayed in supernatants, and the results are expressed as described in Fig. 1. ○, ●, Sialyltransferase activity released from control (○) and 36 h-inflamed (●) rat liver Golgi membranes. The 100 % value represents 2916 units of sialyltransferase activity/mg of Golgi protein. Each point represents the mean result for four experiments, and reproducibility was within ± 12 %.

Table 1. Release of sialyltransferase from Golgi membranes by treatment with ultrasonic vibrations followed by incubation at pH 5.6 and 7.0

Intact Golgi membranes from control and 36 h-inflamed rat livers were incubated for 30 min at pH 5.6 or 7.0, either directly or after treatment with ultrasonic vibration for 30 s. Particulate material was sedimented and sialyltransferase was assayed in the supernatants. Results are means ± S.E.M. from four experiments.

Sample	pH ... Condition ...	Sialyltransferase activity released (units/mg of Golgi membrane protein)			
		7.0		5.6	
		Control	Inflamed	Control	Inflamed
Intact Golgi		36 ± 3	111 ± 10	238 ± 25	646 ± 55
Ultrasonicated Golgi		48 ± 4	234 ± 22	895 ± 70	3087 ± 250

Fig. 3. Effect of pepstatin A on the release of sialyltransferase activities from sonicated Golgi membranes at pH 5.6

Golgi membranes were sonicated, then incubated at pH 5.6 with increasing concentrations of pepstatin A; sialyltransferase was assayed in supernatants, and results are expressed as described in Fig. 1. The 100% value represents 3126 units of sialyltransferase activity/per mg of Golgi protein. ○, ●, Sialyltransferase activity from control (○) and 36 h-inflamed (●) rat liver Golgi membranes. Each point represents the mean for six experiments, and reproducibility was within $\pm 10\%$.

transferase activities were largely recovered in the membranes. Control experiments were carried out in which the release of sialyltransferase from sonicated and unsonicated membranes was compared. Typical results are shown in Table 1 for experiments carried out at pH 5.6 and 7.0. Prior disruption of the vesicles with sonication was essential for the release of sialyltransferase into the supernatant in soluble form. At pH 5.6 the amount of sialyltransferase activity released from sonicated membranes was typically 5 times that found for unsonicated membranes. However, at pH 7.0 there was only a small increase in release of sialyltransferase from sonicated membranes compared with unsonicated controls (Table 1). The period of sonication was also examined. It was found that 30 s of sonication was sufficient to cause maximum release of sialyltransferase from the Golgi membranes when they were subsequently incubated at pH 5.6 (results not shown). Values for apparent K_m for sialyltransferase were determined for the Golgi-membrane-bound enzyme and the enzyme activity released as a result of incubation of sonicated Golgi membranes at pH 5.6. Values of K_m with asialo- α_1 -acid glycoprotein as substrate were found to be 10.0–13.0 μM for the supernatant fractions and 15–19 μM for the bound enzyme; as was found in previous work [1], there was little difference in K_m for the enzyme from

Fig. 4. Effect of addition of antiserum containing antibodies raised against rat liver lysosomal cathepsin D on the release of sialyltransferase from sonicated Golgi membranes

Golgi membranes were sonicated, then incubated at pH 7.0 for 45 min with increasing volumes of antiserum containing antibodies against rat liver lysosomal cathepsin D. The pH was then lowered to 5.6 and samples were incubated for a further 30 min and sialyltransferase was assayed in supernatants as described in Fig. 1. The 100% value represents 3126 units of sialyltransferase activity/mg of Golgi protein. ○, ●, Sialyltransferase activities from control (○) and 36 h-inflamed (●) rat liver Golgi membranes. Each point represents the mean for four experiments, and reproducibility was within $\pm 15\%$.

control or inflamed rat livers. The K_m values for the released enzyme compare favourably with K_m values of 7.1–10 μM for the soluble serum sialyltransferase and 7.6–8.3 μM for sialyltransferase secreted from liver slices [1,6]. The kinetic data supports the idea that the enzyme released from Golgi membranes by sonication and incubation at lowered pH contains the catalytic site. Sialyltransferase in supernatant fractions was also characterized using antiserum containing antibodies against rat hepatic Gal β 1-4GlcNAc α 2-6-sialyltransferase, by using an approach described previously [1,6]; over 90% of the enzyme activity was inhibited in presence of antibodies, showing that the main activity released was the Gal β 1-4GlcNAc α 2-6 enzyme (results not shown).

Characterization of the proteinase that releases sialyltransferase from Golgi membranes

The results described above suggest that sialyltransferase is released from Golgi membranes by the action of an endogenous proteinase which is most effective at pH 5.6. In order to obtain information on the nature of the proteinase activity, proteinase inhibitors

were added before incubation of the sonicated Golgi suspension at pH 5.6 for 30 min (see the Materials and methods section). Sialyltransferase activities were assayed in the supernatant fractions as described above. It was found that pepstatin A was the only inhibitor that had a significant inhibitory effect on release of sialyltransferase from Golgi membranes under the conditions used in the experiments. Fig. 3 shows that pepstatin A was a very effective inhibitor of the release of sialyltransferase from Golgi membranes, with concentrations as low as 10^{-7} M being sufficient to substantially inhibit release of sialyltransferase from control- and inflamed-rat Golgi membranes. The presence of dimethyl sulphoxide used to dissolve the pepstatin A over the range of concentrations of pepstatin A used in Fig. 3 had little or no effect on sialyltransferase activities. Since pepstatin A has been shown to be a very effective inhibitor of cathepsin D [9,26], experiments were performed in which Golgi membranes were incubated with antiserum containing antibodies raised against rat liver lysosomal cathepsin D using an approach described in previous work [6–7]. In these experiments, Golgi membranes from control and inflamed rats were sonicated at pH 7.0 and then incubated with appropriate volumes of antiserum for 45 min; the pH was adjusted to 5.6 and incubation continued for 30 min. Samples used as controls either did not contain antiserum or contained appropriate volumes of normal rabbit serum. Fig. 4 shows that the release of sialyltransferase from Golgi membranes was substantially inhibited by the presence of increasing amounts of antiserum. The presence of normal rabbit serum had no detectable effect on sialyltransferase activities, and enzyme continued to be released in the control experiments where incubation at pH 7.0 in absence of antiserum preceded incubation at pH 5.6. The effect of addition of exogenous bovine spleen cathepsin D on the release of sialyltransferase was also studied. Table 2 shows that the addition of exogenous cathepsin D caused sialyltransferase to be released more rapidly than corresponding samples which did not contain added enzyme. Galactosyltransferase was resistant to proteolytic attack

under these conditions, since most or all of the enzyme activity was recovered in the pellet.

The above results do not provide unequivocal evidence that the proteinase that is responsible for the release of sialyltransferase is present at the luminal side of the Golgi membrane. In order to obtain evidence to support this idea, experiments were performed in which Golgi vesicles from control and 36 h-inflamed rats were incubated at pH 5.6 for 4 and 16 min in presence of 10^{-6} M-pepstatin A; parallel controls were set up in which pepstatin A was not present. Pepstatin A is known to penetrate membranes quite slowly [27], so that it should inhibit mainly extraluminal proteinase activities; the lowered pH would be expected to have an influence on the proton pumps that lower intraluminal Golgi pH

Table 2. Release of sialyltransferase from sonicated Golgi membranes incubated with bovine spleen cathepsin D at pH 5.6

Golgi membranes from control and 36 h-inflamed rat liver were treated with ultrasonic vibration for 30 s and incubated either alone (–) or in presence of 1 µg of bovine cathepsin D/mg of Golgi membrane protein (+); the pH was adjusted to 7.0 to stop the reaction, particulate material was sedimented, and sialyltransferase was assayed in the supernatants. Results are means ± S.E.M. from four experiments.

Incubation time (min)	Rat liver ... Cathepsin D ...	Sialyltransferase activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
		–	+	–	+
2		47 ± 3	80 ± 5	135 ± 12	166 ± 10
6		141 ± 8	222 ± 16	406 ± 30	621 ± 20
12		281 ± 10	466 ± 25	812 ± 55	1144 ± 82

Table 3. Effect of incubation of intact Golgi membranes with pepstatin A followed by sonication to release intraluminal sialyltransferase

Intact Golgi membranes from control and 36 h-inflamed rat livers were incubated for 4 min and 16 min at pH 5.6 in the presence of 10^{-6} M-pepstatin A to inhibit any extraluminal proteinase activity; parallel controls at pH 5.6 and 7.0 in which pepstatin A was absent were set up. At the end of incubation all samples were adjusted to pH 7.0, and 400 µl portions were removed from each sample; this represents the unsonicated (–) sample; the remaining material was sonicated (+) to disrupt the membranes to release any sialyltransferase that was no longer membrane-bound. Particulate material was sedimented, and sialyltransferase was assayed in the supernatants. Under the conditions where pepstatin A was present, sialyltransferase could only have been released from the intraluminal side of the membrane, whereas in the absence of pepstatin A at pH 5.6 sialyltransferase could be released from both sides of the membrane. Results are means ± S.E.M.

Sample	Incubation time (min)	pH ... Sonication ...	Sialyltransferase activity released (units/mg of Golgi membrane protein)					
			7.0		5.6		5.6 + pepstatin A	
			–	+	–	+	–	+
Control	4		22 ± 2	58 ± 5	123 ± 10	327 ± 25	82 ± 7	261 ± 20
	16		41 ± 3	74 ± 6	198 ± 18	406 ± 35	107 ± 10	321 ± 30
Inflamed	4		62 ± 5	159 ± 12	271 ± 20	1270 ± 80	204 ± 21	1077 ± 101
	16		73 ± 7	194 ± 15	565 ± 55	1749 ± 90	431 ± 53	1570 ± 125

Table 4. Cathepsin D activity in intact Golgi membranes and supernatant fractions prepared by sonication, extraction with Triton X-100 and incubation with mannose 6-phosphate

Golgi membranes from livers from control and 36 h-inflamed rats were suspended in 0.25 M-sucrose containing 0.1 M-potassium phosphate buffer (pH 6.5)/5 mM-MgCl₂ at 10 mg of protein/ml. Samples of the suspension were assayed directly for cathepsin D; this represents the intact Golgi. Cathepsin D was also assayed in supernatant fractions prepared by sonication followed by centrifugation together with parallel unsonicated controls, extraction with 0.1% Triton X-100, or sonication followed by incubation with 5 mM-mannose 6-phosphate together with appropriate unsonicated controls (see the Materials and methods section). Supernatants from unsonicated controls contained low levels of cathepsin D activity; values reported below have been corrected for the appropriate control activities. Assays were carried out in duplicate for each sample. Results are expressed as total cathepsin D activity in 1 ml of Golgi suspension and the supernatant fractions derived therefrom; results are means $\pm$ S.E.M. from three separate experiments.

Sample	Cathepsin D activity (units)	
	Control	Inflamed
Intact Golgi	2.50 $\pm$ 0.30	4.04 $\pm$ 0.35
Supernatant fractions		
Sonicated Golgi	0.10 $\pm$ 0.01	0.61 $\pm$ 0.07
Triton X-100	2.42 $\pm$ 0.21	3.57 $\pm$ 0.35
Mannose 6-phosphate	1.90 $\pm$ 0.11	3.17 $\pm$ 0.25

[28,29] to allow intraluminal proteinases to act. After incubation, pH was adjusted to 7.0 as before, and one set of samples from control and inflamed livers were sonicated to disrupt vesicles so as to release any sialyltransferase that was no longer membrane-bound (see the Materials and methods section). Table 3 shows that substantial sialyltransferase was found in the supernatant fractions in which intact Golgi were preincubated at pH 5.6 with pepstatin A and then sonicated. These values were only slightly lower than values from Golgi membranes that were sonicated and incubated at pH 5.6 in the absence of pepstatin A. This suggests that the proteinase is active at the luminal face of the Golgi membrane. To further support this idea, cathepsin D activity was determined in intact Golgi membranes, and in supernatant fractions after sonication, treatment with Triton X-100 or incubation of sonicated Golgi membranes with 5 mM-mannose 6-phosphate. Table 4 shows that Golgi membranes from liver from inflamed rats contained higher levels of cathepsin D activity. However, most of the cathepsin D activity appeared to be membrane-bound, since it could only be released by treatment with Triton X-100 or by sonication and incubation with mannose 6-phosphate to inhibit binding to the mannose 6-phosphate receptor.

Immunoblotting of sialyltransferase

The most likely model to explain the release of a membrane-bound enzyme like sialyltransferase is that the catalytic portion of the enzyme is cleaved from a membrane anchor and this allows the enzyme to be

Fig. 5. Immunoblot analysis of sialyltransferase from intact Golgi membranes from 36 h-inflamed rat liver and sialyltransferase released from Golgi membranes by sonication followed by incubation at pH 5.6

Intact Golgi membranes from 36 h-inflamed rat liver were extracted with Triton X-100 to solubilize the membrane-bound sialyltransferase and compared with sialyltransferase released from Golgi membranes by sonication followed by incubation at pH 5.6 by SDS/polyacrylamide-gel electrophoresis and immunoblot analysis (see the Materials and methods section for details). Track 1, Golgi membrane sialyltransferase; track 2, sialyltransferase released from Golgi membranes by sonication and incubation at pH 5.6. M_r markers are indicated by arrows and were: soybean trypsin inhibitor (T, M_r 21600), carbonic anhydrase (C, M_r 31000), ovalbumin (O, M_r 42699), bovine serum albumin (A, M_r 66200) and phosphorylase B (P, M_r 97400).

secreted from the Golgi into the extracellular space. To obtain information to support this model, Golgi membranes and supernatants after incubation of sonicated membranes at pH 5.6 were examined for the presence of sialyltransferase using the immunoblot procedure. Typical results are shown in Fig. 5. Extracts of Golgi membranes contained a major band with M_r of about 49000, whereas the supernatant contained a trace of this species, but the major component had M_r of about 42000.

DISCUSSION

Gal β 1-4GlcNAc α 2-6-sialyltransferase is a membrane-bound enzyme located at the intraluminal face of the *trans*-Golgi compartment of the hepatocyte, but it is particularly sensitive to release from the liver during the acute-phase response [1,4,6,30,31]. The most likely explanation for the release of a membrane-bound enzyme like sialyltransferase is that it is subject to limited proteolysis, which cleaves the catalytic portion of the enzyme from a membrane anchor. This possibility was investigated in the present work, in which it was found that sialyltransferase, but not galactosyltransferase, could be released from Golgi vesicles by sonication to permeabilize the vesicles followed by lowering of the pH to create an environment where endogenous proteinases could act. About 70% of the Golgi-bound sialyltransferase activity could be released under these conditions, with maximum release occurring after 30 min

incubation at pH 5.6. It was found that release of sialyltransferase activities tended to decline at longer incubation times, presumably because of increased proteolytic degradation of the enzyme; the same explanation could account for the lower activities of sialyltransferase released from the Golgi at pH values below 5.6 when proteinase activities are likely to be enhanced.

The immunoblot procedure was used to characterize the bound and released forms of sialyltransferase. The main band from the Golgi-bound sialyltransferase had an M_r of 49000, which is comparable with the main species of sialyltransferase found in liver by Paulson's group, which had an M_r of 47000 [23,24,32,33]. Sialyltransferase released from the Golgi by the action of the endogenous proteinase had an M_r closer to 42000. The nucleotide sequence of cDNA clones of Gal β 1-4GlcNAc α 2-6-sialyltransferase has recently been reported by Paulson's group [32-34]. These workers showed that sialyltransferase contains a signal sequence and a membrane-anchor domain at the *N*-terminus; the membrane anchor including the signal sequence corresponded to 63 amino acid residues. The catalytic portion of the enzyme could be removed from this segment, giving an enzyme with full catalytic activity and an M_r of 41000 [32-34]. The observations reported in the present study are consistent with the model described by Paulson [32-34]. It is likely that the 42000- M_r form of sialyltransferase released from the Golgi membranes by the action of an endogenous proteinase (Fig. 5) corresponds to the catalytic unit described above which has been cleaved from the 63-amino-acid segment containing the membrane anchor.

The release of sialyltransferase into the extracellular space by an endogenous proteinase is clearly of great importance in understanding the physiological events occurring in the liver during the acute-phase response. For this reason an attempt was made to characterize the proteinase and to determine the location of the enzyme in the Golgi vesicle. Release of sialyltransferase from permeabilized Golgi vesicles was significantly inhibited by the presence of pepstatin A, a potent inhibitor of cathepsin D-like aspartic proteinases; other proteinase inhibitors studied had little or no effect on the release of sialyltransferase from Golgi vesicles. The cathepsin D-like nature of the proteinase was confirmed by inhibition studies utilizing antiserum raised against lysosomal cathepsin D coupled with experiments in which it was shown that the addition of exogenous cathepsin D caused enhanced release of sialyltransferase from the Golgi vesicles. Experiments were designed to determine whether the proteinase was located on the luminal face of the Golgi vesicle. In these experiments Golgi vesicles were incubated at pH 5.6 in the presence of pepstatin A on the premise that, if sialyltransferase was released from the luminal face of the membrane, then the proteinase would have to be active at the luminal side. Vesicles were sonicated after incubation to permeabilize the membranes to release any sialyltransferase that was cleaved in the intraluminal space. The presence of pepstatin A in the incubation mixture before permeabilization would inhibit any residual proteinase that might have been released during resuspension or was exposed on 'inside-out' vesicles in the preparation. Sialyltransferase on inside-out vesicles would be released under these conditions in the absence of pepstatin A, which explains the higher sialyltransferase activities released at pH 5.6 alone com-

pared with the pH 5.6 samples in the presence of pepstatin A (Table 3). Substantial sialyltransferase activity was released from vesicles incubated at pH 5.6 in presence of pepstatin A. Since this could only have come from the intraluminal face, it provides strong evidence that the proteinase is active at the luminal side of the membrane.

Proteinases endogenous to the Golgi membranes have been attributed with the cleavage of propeptides from proteins such as proalbumin [35], and it is well known that processing of prohormones occurs in cells in a variety of intracellular compartments [36-39]. It is not surprising that a proteinase is located in the Golgi membranes that can cleave a membrane-bound enzyme like sialyltransferase. However, it is not clear if the cathepsin D-like enzyme described in the present work is specifically charged with the task of cleaving sialyltransferase, or if it represents a lysosomal proteinase, most likely cathepsin D, which has found its way into the compartment where sialyltransferase is located and is acting on the enzyme. The precise intracellular site where the final sorting of lysosomal enzymes and secretory proteins occurs has not been identified unequivocally. However, current reports suggest that a *trans*-Golgi network ('TGN') located close to the *trans*-compartments of the Golgi may be involved [41,42]. The *trans*-Golgi network may also represent the intracellular site where Gal β 1-4GlcNAc α 2-6-sialyltransferase is located [8,41,42]. The release of sialyltransferase could come about as a result of inefficient targeting of lysosomal cathepsin D, resulting in some catalytically active enzyme passing into the secretory compartments of the cell where sialyltransferase is located, resulting in the release of the enzyme from the Golgi membrane. This idea is supported by the higher cathepsin D activities found in Golgi membranes from livers from inflamed rats (Table 4). The cathepsin D also appeared to be mainly membrane-bound, since Triton X-100 was required for efficient release of the enzyme; moreover, the results showing that mannose 6-phosphate could release substantial cathepsin D from sonicated membranes supports the idea that the enzyme is located at the luminal face of the Golgi [16,40,41]. Inefficient targeting of lysosomal cathepsin D represents a valid model to explain the release of sialyltransferase, since lysosomal enzymes are known to be secreted, particularly under acute-phase conditions [3], and it has been shown that the receptor for the mannose 6-phosphate signal that targets lysosomal enzymes to the lysosome is present in the *trans*-Golgi compartment of the cell [40,41], possibly in the same compartment where sialyltransferase is present. Also, it has been shown that Golgi membranes possess proton pumps which produce intra-Golgi pH values of about 6 [28,29]; indeed, lowered intra-Golgi pH may be a prerequisite for sorting of proteins during passage through the Golgi [40-42]. Thus conditions could exist where lysosomal cathepsin D or an endogenous proteinase with cathepsin D-like activity would be active under physiological conditions. Indeed, it is likely that release of sialyltransferase from liver occurs under normal conditions, since the enzyme is present in low levels in normal mammalian serum. However, it is clear that the enzyme is released in increased amounts during the acute-phase response, and this seems to represent a specific release of this enzyme, since galactosyltransferase, another Golgi located enzyme, is not released under the same conditions.

An understanding of the factors controlling the release

of sialyltransferase from the Golgi membranes is of great importance in explaining the acute-phase response of sialyltransferase in a wide range of inflammatory conditions in humans, including neoplastic disease and rheumatoid arthritis. Further studies are required in order to characterize the cathepsin D-like proteinase identified in the present study and to determine its physiological role in the acute-phase response.

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