Asparagine-linked glycoprotein biosynthesis in rat epididymis

Presence of a mannosidase II-like enzyme

Marjorie D. SKUDLAREK, Marie-Claire ORGEBIN-CRIST and Daulat Ram P. TULSIANI*

Center for Reproductive Biology Research, Department of Obstetrics and Gynecology, Vanderbilt School of Medicine, Nashville, TN 37232–2633, U.S.A.

Previous studies from this laboratory using *p*-nitrophenyl α -D-mannoside (*p*-NPM) as substrate provided no evidence for the presence of mannosidase II in the rat epididymis [Skudlarek & Orgebin-Crist (1988) *J. Reprod. Fertil.* **84**, 611–617]. However, rat epididymal epithelial cells cultured in the presence of swainsonine, an inhibitor of mannosidase II, produce abnormally processed *N*-linked glycoproteins containing hybrid-type oligosaccharides instead of complex-type [Tulsiani, Skudlarek & Orgebin-Crist (1990) *Biol. Reprod.* **43**, 130–138], a result providing indirect evidence for the presence of mannosidase II-like enzyme in rat epididymis. In the studies described here, we present evidence for the occurrence of this processing enzyme in rat epididymal Golgi membranes. This enzyme is an integral Golgi membrane component. Like liver mannosidase II, the epididymal enzyme cleaves α 1,3- and α 1,6-linked mannosyl residues from GlcNAcMan₅GlcNAc. However, unlike liver mannosidase II, the epididymal enzyme shows no activity towards the synthetic substrate, *p*-NPM. The epididymal mannosidase cross-reacts with liver anti-(mannosidase II) antibody, a result suggesting that the two enzymes share a common antigenic site(s). Immunoblotting studies following resolution of liver and epididymal Golgi membranes on SDS/PAGE show that, whereas the liver mannosidase II was resolved as a doublet of *M_r* 120 000 and 122 000, only the *M_r* 120 000 band was observed in the epididymal Golgi membranes. Immunoblotting of the Golgi-rich fractions, resolved under non-denaturing conditions, showed different patterns of charge and/or size isomers from the two tissues. These studies demonstrate tissue-specific differences in processing enzymes with similar function.

INTRODUCTION

Mannosidase II, the enzyme involved in *N*-linked glycoprotein biosynthesis, has been purified to homogeneity from rat liver Golgi membranes and chemically characterized [1–3]. This enzyme is active towards *p*-nitrophenyl α -D-mannoside (*p*-NPM) and oligosaccharide (GlcNAcMan₅GlcNAc-) substrates, with a pH optimum of 5.2–5.8 [2]. The activities towards the synthetic and physiological substrates behave very similarly in that they (1) purify in a parallel manner [2], (2) are inactivated at the same rate [2,4] and (3) respond similarly towards several effectors [2,4]. These results support the conclusion that the activities towards both *p*-NPM and GlcNAcMan₅GlcNAc- substrates are due to mannosidase II [2].

Previous studies have provided no evidence for the presence of mannosidase II in the epididymis [5] and brain of several species [6,7]. However, rat brain microsomal fraction contains an α -D-mannosidase capable of converting high-mannose oligosaccharides (Man₅GlcNAc-Man₅GlcNAc) into the trimannosyl core structure (Man₃GlcNAc) found in complex glycoproteins [6]. This enzyme could replace several known mannosidases in the formation of mature complex glycoproteins in the brain. Rat epididymal tissue contained no such mannosidase activity [8]. Since epididymis-mediated modifications of sperm surface glycoproteins are believed to be important for the successful interaction of gametes, it was of interest to identify and characterize glycoprotein-processing enzymes in this tissue. In this report, we present evidence for the occurrence of a mannosidase II-like enzyme in rat epididymal Golgi membranes. The epididymal enzyme, like liver mannosidase II, cleaves α 1,3- and α 1,6-linked mannosyl residues from GlcNAcMan₅GlcNAc. However, unlike mannosidase II, the epididymal enzyme shows no activity towards *p*-NPM.

MATERIALS AND METHODS

Chemicals and antibodies

Sprague–Dawley rats (28–32-day-old) obtained from Sasco, Omaha, NE, U.S.A. were kept in our animal facilities for at least 3 days before experiments were begun. Animals were killed by CO₂ asphyxiation. Each epididymis was dissected free from the fat-pad. Most of the visible blood vessels were removed under a dissecting microscope while the tissue was kept on ice (0–4 °C). Uniformly labelled oligosaccharides were prepared as described previously [9]. Swainsonine, isolated from *Rhizoctonia leguminicola* [10], was provided by Dr. H. P. Broquist of this University. UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-galactose (UDP-Gal) were from Sigma Chemical Co.; labelled nucleotide sugars, namely UDP-[6-³H(n)]GlcNAc and UDP-[U-¹⁴C]Gal were from DuPont Co. (Wilmington, DE, U.S.A.); Vectastain reagents were from Vector Laboratories (Burlingame, CA, U.S.A.); all electrophoresis and electroblotting chemicals were from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals used were obtained commercially and were of the highest purity available. Polyclonal antibody (rabbit IgG fraction) against rat liver Golgi mannosidase IA was prepared as described previously [11]. Anti-(rat liver mannosidase II) IgG was prepared as described in ref. [3]. Polyclonal antibody (IgG fraction) against rat epididymal (lysosomal) α -D-mannosidase was prepared as reported previously [12]. The 0.4 M-salt-washed Golgi-rich fraction was prepared from liver of 31–35-day-old rats as previously described [1].

Preparation of epididymal Golgi membranes

The method is similar to a previously published procedure for rat liver [1], with several modifications. Briefly, the tissue was mixed with 0.5 M-sucrose solution (4 ml/g wet wt.) at 0–4 °C. All

Abbreviations used: *p*-NPM, *p*-nitrophenyl α -D-mannoside; PBS, phosphate-buffered saline; PCMPS, *p*-chloromercuriphenylsulphonic acid.

* To whom correspondence should be addressed.

the sucrose solutions used in the procedure contained 0.05 M-sodium maleate buffer, pH 6.5, and 5 mM-MgCl₂. The tissue was homogenized for 50 s with a Polytron homogenizer (type PT 10 20 3500; Brinkman Instruments) set at position 5, and centrifuged at 800 *g* for 10 min. The supernatant was removed by aspiration and the residue was homogenized and centrifuged as above. This process was repeated once more. Pooled supernatant (cytoplasmic extract) (4 ml) was layered on top of 11.5 ml of 1.3 M-sucrose in an ultra-clear 16 mm × 102 mm centrifuge tube (Beckman Instruments) and the gradient was centrifuged at 120 000 *g* for 90 min in an SW 28.1 rotor. The membranes accumulating on top of the 1.3 M-sucrose were collected by aspiration (first gradient). After the sucrose concentration was adjusted to 1.1 M, the membranes (5 ml) were layered on top of 7 ml of 1.25 M-sucrose, followed by 4 ml of 0.5 M-sucrose. The gradient was centrifuged at 120 000 *g* for 90 min. The membranes accumulating as a white band at the interphase between 0.5 M- and 1.1 M-sucrose solution were collected by aspiration (second gradient) and used to prepare Golgi-rich fraction. The pinkish-looking membranes immediately below the white band were collected separately, diluted with 4 vol. of phosphate-buffered saline (PBS; prepared by our published procedure [13]), and centrifuged at 105 000 *g* for 30 min in a 50 Ti rotor (Beckman Instruments). The resulting pellet (crude membranes) was used for biochemical and ultrastructural studies. The white membranes from the second gradient (see above) were adjusted to 1.1 M-sucrose. A 3 ml portion of the membrane suspension was layered on top of 8 ml of 1.25 M-sucrose, followed by 5.5 ml of 0.5 M-sucrose. Tubes were centrifuged at 120 000 *g* for 90 min in an SW 28.1 rotor. The Golgi membranes accumulated as a dense band at the interphase between 0.5 M- and 1.1 M-sucrose solution. The band was collected by aspiration (third gradient). The sucrose concentration was lowered to 0.25 M by diluting with ice-cold distilled water, the salt concentration was adjusted with 1 M-NaCl to 0.4 M, and the membranes were pelleted by centrifugation at 105 000 *g* for 30 min. The resulting Golgi-rich membranes were used for biochemical and ultrastructural studies.

Preparation of epididymal particulate fraction

The tissue homogenate was prepared in 0.25 M-sucrose containing 5 mM-Tris/HCl buffer, pH 8.0 (4 vol./g of tissue) by homogenizing for 50 s with a Polytron homogenizer (see above) set at position 5. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was removed by aspiration and the residue was homogenized and centrifuged as above. This process was repeated a third time. The particulate fraction was obtained by centrifuging the pooled cytoplasmic extract at 105 000 *g* for 30 min.

Solubilization of membrane fractions

The epididymal particulate fraction was solubilized by repeated extraction in potassium phosphate buffer (10 mM, pH 7.2) containing 0.5 % Triton X-100, 0.25 % deoxycholate and 0.25 M-NaCl as described in ref. [8]. The Golgi-rich fraction (from epididymis and liver) was solubilized by suspension in the above phosphate buffer containing 2 % Triton X-100 and 1.0 % sodium deoxycholate, followed by homogenization in a glass homogenizer with a Teflon pestle rotating at 1200 rev./min (five up and five down strokes). The suspension was centrifuged at 165 000 *g* for 30 min and the supernatant was removed by aspiration. The pellet was resuspended in a small volume of the above buffer, again homogenized and centrifuged as above. The combined supernatant was designated as solubilized membrane fraction.

Ultrastructural studies

The isolated Golgi-rich membranes and crude membranes were fixed with 4 % glutaraldehyde in 0.1 M-sodium cacodylate buffer at 4 °C as described in ref. [14]. Thin sections, stained with uranyl acetate and lead citrate, were examined in a Hitachi H-600 electron microscope.

Immunoprecipitation studies

The appropriate antibody was pre-adsorbed to the washed IgG-sorb [15] by incubating 100 µl of the IgG-sorb with the antibody (7 mg of protein/ml) at room temperature for 60 min with occasional mixing. Excess of the antibody was removed by suspending the mixture in washing buffer [potassium phosphate buffer (25 mM, pH 7.5) containing 2 % Triton X-100, 0.5 % sodium deoxycholate and 0.25 M-NaCl] and centrifugation in an Eppendorf microcentrifuge for 1 min (three washes). The pellet was mixed with a small volume (30–50 µl) of the solubilized membrane fraction, and the mixture was kept at room temperature for 60 min with occasional mixing. The supernatant obtained after centrifugation was assayed for enzyme activities. However, if the antibody–antigen complex was used for enzyme assay, the pellet left after removing the supernatant was washed twice by suspension in 0.5 ml of the washing buffer and centrifugation (see above). The pellet (immobilized antibody–antigen complex) was suspended in the desired buffer and used to quantify the antigen.

Analytical gel electrophoresis

Polyacrylamide-gel electrophoresis under non-denaturing conditions was carried out in the slab-gel electrophoresis system of Davis [16]. The solubilized sample in 10 % sucrose was layered on top of a 3 %-acrylamide stacking gel (1.5 mm-thick), and resolved on a 7 % gel with Triton X-100 (0.2 %, v/v) present in the gels and electrophoretic buffer as described elsewhere [17]. The system of Laemmli [18] was used to resolve the proteins under denaturation conditions. The Golgi membranes were solubilized by mixing in SDS buffer containing 10 % dithiothreitol and placing the tube in boiling water for 5 min. The heat-treated sample was centrifuged in a microcentrifuge for 2 min and the solubilized proteins were removed by aspiration. Proteins were resolved on a 1.5 mm-thick polyacrylamide gel for 3–4 h at 40 mA/gel with cooling at 8 °C.

Electrotransfer to nitrocellulose paper and immunostaining

Proteins and polypeptides were electrophoretically transferred to 0.45 µm-pore nitrocellulose sheets as described by Towbin *et al.* [19]. The transfer was conducted for 2 h at 300 mA in transfer buffer cooled at 4 °C. After transfer, the nitrocellulose paper was immersed in PBS containing 1 % BSA, and left in the solution overnight at 0–4 °C. All remaining procedures were done at room temperature. The paper was washed three times (5 min each) in the above BSA solution containing 0.1 % Tween. Immunodetection was done by incubating in anti-(mannosidase II) antibody (diluted 750-fold in the above PBS solution). After 60 min, excess of the antibody was removed by three PBS washes. The washed nitrocellulose paper was incubated with goat anti-(rabbit IgG) (Vectastain ABC kit PK 400, Vector Laboratories) at 1:750 dilution in PBS for 60 min. The paper was washed six times in PBS and incubated with Vectastain reagent for 30 min. The paper was washed four times in PBS, and the immune complex was detected with diaminobenzidine solution (2.4 mM) prepared in 0.1 M-Tris/HCl buffer, pH 7.6. The reaction was stopped by rinsing the paper with distilled water.

Enzyme assays

p-Nitrophenyl mannosidase activity was quantified by measuring the release of *p*-nitrophenol in a standard incubation mixture (0.5 ml) containing 4 mM-*p*-NPM, 0.2% Triton X-100 and the desired buffer as described [20]. After incubation for 1–4 h at 37 °C, the reaction was stopped by addition of 1.0 ml of an alkaline buffer adjusted to pH 10.7. The *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm. Enzyme and substrate blanks were run with all assays. One unit is the amount of enzyme that catalyses the release of 1 μ mol of *p*-nitrophenol/min.

Oligosaccharide-cleaving mannosidase activity was assayed by measuring the hydrolysis of [³H]mannose-labelled oligosaccharide in a standard incubation mixture (50 μ l) containing radiolabelled substrate (about 4000 c.p.m.) and the desired buffer (0.1 M). After incubation for 1–4 h at 37 °C, the reaction was stopped by placing the tube in boiling water (5 min). The released [³H]mannose was separated from the labelled oligosaccharide by gel filtration and quantified as described previously [2]. One unit is the amount of enzyme that catalyses the release of 1000 c.p.m. of [³H]mannose/h at 37 °C.

Galactosyltransferase was assayed by measuring the amount of [¹⁴C]galactose transferred to the exogenous acceptor (asialo-agalacto-ovomucoid) by the procedure of Bretz *et al.* [21] as described in ref. [13]. *N*-Acetylglucosaminyltransferase (GlcNAc transferase) was assayed by measuring the amount of [³H]glucosamine transferred to the exogenous acceptor (asialo-agalacto-aglucosamino-ovomucoid) by the procedure described previously [21]. One unit of galactosyltransferase/GlcNAc transferase is the amount of enzyme that catalyses the transfer of 1 nmol of monosaccharide/min.

Phosphodiesterase I, a known liver plasma-membrane marker enzyme [22], was assayed by measuring the release of *p*-nitrophenol in a standard assay mixture (0.5 ml) containing 1 mM-*p*-nitrophenyl 5'-thymidylate, 50 mM-Tris/HCl buffer and 0.2% Triton X-100 at pH 9.0. After incubation at 37 °C for 15–60 min, the reaction was stopped by addition of 1 ml of an alkaline buffer, containing 0.133 M-glycine, 0.083 Na₂CO₃ and 0.067 M-NaCl, adjusted to pH 10.7 with NaOH [23]. The *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm. One unit is the amount of enzyme that catalyses the release of 1 μ mol of *p*-nitrophenol/min.

Glucosidase II, a marker enzyme for rat liver endoplasmic reticulum [24,25], was assayed by measuring the hydrolysis of *p*-nitrophenyl α -D-glucoside. The standard assay mixture contained 50 mM-sodium citrate/sodium phosphate buffer, pH 6.5, 5 mM-substrate and enzyme in a total volume of 0.5 ml [26]. After incubation for 30–60 min at 37 °C, the reaction was stopped by adding 1.0 ml of the alkaline buffer, pH 10.7 (see above). The *p*-nitrophenol released was estimated by measuring the absorbance at 400 nm. One unit is the amount of enzyme that catalyses the release of 1 nmol of *p*-nitrophenol/min.

Protein was assayed by the fluorimetric method of Anderson & Desnick [27] with BSA as standard.

RESULTS

Evidence for the presence of GlcNAcMan₅GlcNAc-cleaving mannosidase activity in the rat epididymis

Since earlier studies indicated the presence of a novel α -D-mannosidase in the sperm plasma membrane [8], with a broad substrate specificity, cleaving α 1,2-linked mannosyl residues from Man₅GlcNAc and Man₆GlcNAc, as well as α 1,3-(α 1,6)-linked mannosyl residues from GlcNAcMan₅GlcNAc, it was desirable to prepare membrane fraction from sperm-free epididymal tissue.

The particulate fraction from sexually immature rat epididymis was solubilized by repeated extraction with a detergent/salt solution. The solubilized fraction contained > 95% of the activities towards *p*-NPM or GlcNAcMan₅GlcNAc substrate, when assayed at pH 4.5 or 6.0. The more acidic value is the pH optimum for lysosomal α -D-mannosidase [12] whereas the less acidic buffer is close to the pH optimum for the liver Golgi mannosidase II [1,2]. Results from these studies, summarized in Table 1, show the following. (1) The solubilized fraction hydrolyses both the synthetic and oligosaccharide substrates. However, comparison of the two activities at pH 6.0 and 4.5 shows that, whereas the oligosaccharide-cleaving activity was much higher at pH 6.0, the synthetic substrate was hydrolysed similarly at the two pH values. (2) The activities towards the two substrates were differentially immunoprecipitated. The *p*-nitrophenyl mannosidase activity was quantitatively immunoprecipitated with anti-[rat epididymal (lysosomal) α -D-mannosidase] IgG, which had no effect on the oligosaccharide-cleaving activity. (3) The activity towards GlcNAcMan₅GlcNAc showed no cross-reactivity with antibody to liver Golgi mannosidase IA, but was immunoprecipitated with anti-(rat liver Golgi mannosidase II) IgG (Table 1). These preliminary studies provide evidence for the presence of a novel GlcNAcMan₅GlcNAc-cleaving enzyme (mannosidase II-like activity) in the epididymis.

Subcellular localization of mannosidase II-like enzyme activity in rat epididymis

Since rat liver mannosidase II is a Golgi marker enzyme [1,2], it was decided to determine the subcellular localization of mannosidase II-like activity in rat epididymis. Subcellular fractions, namely Golgi-rich fraction, and crude membrane

Table 1. Assay for α -D-mannosidase activities in rat epididymal particulate fraction

Treatment	Mannosidase activity (units/g of tissue)	
	<i>p</i> -NPM	GlcNAcMan ₅ GlcNAc
Solubilized particulate fraction*	27.4 (4.5) 21.8 (6.0)	3.3 (4.5) 19.5 (6.0)
Anti-[rat epididymal (lysosomal) mannosidase] IgG†	ND‖ (4.5) ND‖ (6.0)	3.1 (4.5) 18.9 (6.0)
Anti-(rat Golgi mannosidase I) IgG‡	ND‖ (4.5) ND‖ (6.0)	3.0 (4.5) 16.8 (6.0)
Anti-(rat Golgi mannosidase II) IgG§	ND‖ (4.5) ND‖ (6.0)	0.2 (4.5) 3.3 (6.0)

* Particulate fraction prepared from the epididymis of 31–34-day-old rats was solubilized by repeated extraction with a salt/detergent solution as described in the Materials and methods section. Portions were assayed for the mannosidase activities. The pH of the assay mixture is shown in parentheses.

† The enzyme activities were assayed in the supernatant after immunoprecipitation with anti-[rat epididymal (lysosomal) mannosidase] IgG.

‡ The supernatant left after immunoprecipitation of the lysosomal mannosidase activity (see above) was incubated with IgG sorb that had been preadsorbed with anti-(rat Golgi mannosidase IA) IgG. The enzyme activities were assayed in the supernatant.

§ The supernatant after immunoprecipitation of lysosomal and mannosidase I activities (see above) was incubated with IgG sorb that had been preadsorbed with anti-(rat Golgi mannosidase II) IgG. Enzyme activities were assayed in the supernatant. For other details see the Materials and methods section.

‖ Not detectable.

Fig. 1. Electron micrograph of rat epididymal Golgi-rich fraction (a) and crude membrane fraction (b)

The membrane fractions were prepared from the epididymis of sexually immature rats as described in the Materials and methods section. The membranes were pelleted by ultracentrifugation at 105 000 *g* for 30 min and the pelleted membranes used for ultrastructural studies. Bar: 0.36 μ m.

fraction were prepared as described in the Materials and methods section. Examination of the Golgi-rich fraction by electron

microscopy showed the presence of membrane elements typical of Golgi stack. In addition, numerous vesicles of different size and shape are recognizable, some of which are probably derived from plasma membranes (Fig. 1a). The crude membrane fraction contains membrane vesicles of several morphological classes, including rough and smooth endoplasmic reticulum, plasma membranes and a few elements typical of the Golgi stack (Fig. 1b).

The data presented in Table 2 show that the specific activity for the mannosidase II-like enzyme, like the glycosyltransferases, is much higher in the Golgi membranes than in the cytoplasmic extract and crude membranes. Although the two membrane preparations contained phosphodiesterase I and glucosidase II, the marker enzymes for plasma membranes and endoplasmic reticulum respectively, the crude membranes showed 2-fold higher specific activity for phosphodiesterase I than the Golgi-rich fraction (Table 2). Similarly, the specific activity for glucosidase II is over 2-fold higher in the crude membrane fraction, which contained very little of the mannosidase II-like enzyme activity. On the basis of these results, we conclude that the mannosidase II-like enzyme activity is localized in the epididymal Golgi membranes.

Kinetic properties of epididymal Golgi mannosidase

The rate of hydrolysis of GlcNAcMan₅GlcNAc was directly proportional to Golgi membrane concentration up to at least 60 μ g of membrane protein. With 20–60 μ g of the membrane protein, the free [³H]mannose release was linear for at least 2 h at 37 °C (see below).

Rat liver Golgi mannosidase II has been shown to cleave both the synthetic substrate (pH optimum 5.5) and physiological substrate (pH optimum 5.8). The data presented in Fig. 2 confirm these earlier studies with the liver Golgi membranes [1,2,28]. However, similar studies carried out with the rat epididymal Golgi membranes show that the mannosidase II-like enzyme cleaves only GlcNAcMan₅GlcNAc-, and that the maximum cleavage occurred between pH 5.8 and 6.0 (Fig. 2). Although the epididymal Golgi membranes showed some hydrolysis of *p*-NPM substrate between pH 4.0 and 6.5, this activity was due to the presence of lysosomal α -D-mannosidase as a contaminant, which was quantitatively immunoprecipitated with the anti-[rat epididymal (lysosomal) α -D-mannosidase] antibody. This had no effect on GlcNAcMan₅GlcNAc-cleaving activity (Fig. 2).

Substrate specificity of mannosidase II-like enzyme

To investigate the substrate specificity of the epididymal Golgi mannosidase, the enzyme was first immunoprecipitated from the solubilized Golgi membranes (see above). The immobilized antigen-antibody complex was washed with salt/detergent solution to ensure the removal of other mannosidase activities localized in rat liver endoplasmic reticulum [29,30] and liver Golgi membranes [2,31]. The antigen-antibody complex was incubated with [³H]mannose-labelled oligosaccharides and the released [³H]mannose quantified. It is obvious from the results in Table 3 that the epididymal enzyme shows little or no activity towards substrates containing terminal α 1,2-linked mannosyl residues, as in Man₆GlcNAc, Man₅GlcNAc, Man₇GlcNAc and Man₈GlcNAc. The enzyme cleaves α 1,3- and α 1,6-linked mannosyl residues from Man₆GlcNAc only after the oligosaccharide is glucosaminylated. However, when the terminal *N*-acetylglucosamine is substituted with galactose, as in GalGlcNAcMan₅GlcNAc, the enzyme failed to recognize and cleave the substrate (Table 3). These results confirm our previous studies with rat

Table 2. Comparison of levels of various marker enzymes in preparations of crude membranes and Golgi-rich membranes from rat epididymis

Epididymis (2.36 g) was obtained from sexually immature (31–34-day-old) rats. Membranes accumulating at the interface of 0.5–1.1 M-sucrose (after second sucrose-gradient centrifugation) were removed by aspiration and used to purify Golgi membranes. However, the pinkish-looking membranes present in 1.1 M-sucrose and at the interface of 1.1–1.25 M-sucrose were removed by aspiration. Ice-cold water was added to adjust the sucrose concentration to 0.25 M, and the membranes were pelleted by centrifugation at 165000 *g* for 30 min. The pellet was suspended in the desired buffer (crude membranes) and used for the enzyme and protein assay. Membranes obtained after the third sucrose-gradient centrifugation are the Golgi membranes. Recovery is based on the amount present in the cytoplasmic extract.

Enzyme	Cytoplasmic extract	Crude membranes		Golgi membranes	
	Specific activity (unit/mg of protein)	Specific activity (units/mg of protein)	Recovery (%)	Specific activity (units/mg of protein)	Recovery (%)
Galactosyltransferase	0.52	0.7	11.3	10.1	53.3
GlcNAc transferase	0.12	0.1	6.3	2.1	51.2
Phosphodiesterase I	0.05	0.2	37.0	0.1	9.7
Glucosidase II	0.62	1.6	20.4	0.6	2.7
Mannosidase II-like activity	0.92	1.1	10.3	11.1	33.4*
Protein (mg)	177.2	14.9	8.4	4.9	2.8

* The lower recovery for the mannosidase II-like activity in the Golgi membranes (compare 33.4% with > 50% for the glycosyltransferases) was expected, since the soluble α -D-mannosidase present in cytoplasmic extract is known to hydrolyse GlcNAcMan₆GlcNAc substrate [48].

Fig. 2. Influence of pH on mannosidase activities present in the Golgi-rich fractions

Salt-washed Golgi membranes from rat liver (a) and epididymis (b) were solubilized by repeated extraction with the detergent/salt solution and centrifugation. Portions were incubated with 4 mM-*p*-NPM (○, ●) or about 4000 c.p.m. of GlcNAcMan₆GlcNAc- (Δ, ▲) before (●, ▲) and after (○, Δ) immunoprecipitation of lysosomal (acid) α -D-mannosidase. The reaction buffers were 0.1 M-sodium citrate (pH 3.5), 0.1 M-sodium acetate (pH 4.0–5.8) and 0.1 M-sodium cacodylate (pH 6.0–8.0).

liver Golgi mannosidase II [32] and provide evidence that *N*-acetylglucosamine on the non-reducing terminus is required for the recognition and binding of the epididymal enzyme before the mannosyl residues are cleaved.

The time course of hydrolysis of GlcNAcMan₆GlcNAc- by the mannosidase II-like enzyme is shown in Fig. 3. The reaction was

Table 3. Substrate specificity of rat epididymal mannosidase II-like enzyme

Uniformly labelled oligosaccharides were prepared as described in the Materials and methods section. Solubilized epididymal Golgi-rich fraction (approx. 120 μ g of enzyme protein) was incubated with washed IgG-sorb that had been preadsorbed with anti-(rat liver Golgi mannosidase II) antibody (rabbit IgG fraction). The mixture was kept at room temperature for 90 min with occasional mixing. The immobilized antibody-antigen complex was washed three times by suspension in 0.25 ml portions of 10 mM-potassium phosphate buffer, pH 7.4, containing 1% Triton X-100, 0.25 M-NaCl and 0.25% sodium deoxycholate, and centrifugation in a micro-centrifuge. The washing ensures the removal of mannosidase activities (i.e. endoplasmic-reticulum α -D-mannosidase and Golgi mannosidase I) non-specifically bound to the IgG-sorb. The washed antibody-antigen complex was suspended in 25 μ l of 100 mM-sodium acetate buffer, pH 6.0, mixed with approx. 4000 c.p.m. of the appropriate substrate and incubated at 37 °C for 4 h. The reaction was stopped by heat inactivation, and the contents were extracted three times by suspension in 40 μ l portions of 0.1 M-acetic acid and centrifugation for 2 min. Free [³H]mannose was separated from the oligosaccharide by applying the pooled supernatant to a column of Bio-Gel P-2 and quantified as described in ref. [2]. Since the *K_m* for these substrates is not known, comparative studies were done by using the same amount of radioactivity (c.p.m.) for each oligosaccharide and longer incubation time. Under these conditions only GlcNAcMan₆GlcNAc- shows appreciable hydrolysis.

Substrate	Extent of hydrolysis	
	(% of [³ H]mannose released)	Relative activity (%)
Man ₆ GlcNAc	1.2	6.3
Man ₅ GlcNAc	2.1	11.1
Man ₇ GlcNAc	1.9	10.1
Man ₆ GlcNAc	1.5	7.9
Man ₅ GlcNAc	0.9	4.8
GlcNAcMan ₆ GlcNAc	18.9	100.0
GalGlcNAcMan ₆ GlcNAc	1.5	7.9

linear for up to 2 h, when nearly 20% of [³H]mannose (one mannosyl residue) was cleaved. However, the epididymal enzyme took several hours to release the remaining [³H]mannose. These data provide evidence suggesting that, like liver mannosidase II,

Fig. 3. Time course of hydrolysis of GlcNAcMan₅GlcNAc by epididymal Golgi membranes

Salt-washed Golgi membranes were suspended in 0.4% Triton X-100. Portions containing 50 µg of Golgi membrane protein were incubated at 37 °C in a standard assay mixture (50 µl) containing 0.1 M-sodium acetate buffer, pH 6.0, and approx. 4000 c.p.m. of GlcNAcMan₅GlcNAc-. At the indicated time-interval, the reaction was stopped by heat inactivation (5–7 min in boiling water). Released [³H]mannose was separated on a column of Bio-Gel P-2 and quantified [2].

the epididymal enzyme preferentially hydrolyses either the α1,3- or the α1,6-linked mannose.

Effectors of mannosidase II-like enzyme

Several bivalent cations have previously been shown to have an inhibitory/stimulatory effect on mammalian mannosidases [1,2,8,31]. The data presented in Table 4 show that Cu²⁺ is a potent inhibitor of the epididymal mannosidase II-like enzyme. The other bivalent cations tested had little or no effect on the enzyme. Similarly, the chelating agent EDTA and the thiol reagents mercaptoethanol and *p*-chloromercuriphenylsulphonic acid (PCMPS) had no effect on GlcNAcMan₅GlcNAc-cleaving activity of the epididymal Golgi membranes. The negative effect of PCMPS was surprising, since the reagent at 1 mM concentration has been shown to be a potent inhibitor of *p*-nitrophenyl mannosidase activity of purified liver Golgi mannosidase II [1]. To investigate whether the *p*-NPM- and GlcNAcMan₅GlcNAc-cleaving activities of liver Golgi mannosidase II are differentially inhibited by PCMPS, we studied the effects of different concentrations of the reagent on these two activities of liver Golgi membranes and GlcNAcMan₅GlcNAc-cleaving activity of epididymal Golgi membranes. Data in Fig. 4 show that, whereas PCMPS at 1 mM concentration inhibited nearly 80% of *p*-nitrophenyl mannosidase activity of the liver Golgi mannosidase II, the reagent at the same concentration had little effect on GlcNAcMan₅GlcNAc-cleaving activities of the liver and epididymal Golgi membranes. At higher concentrations, the reagent caused some inhibition of the oligosaccharide-cleaving activity. However, the inhibition curves obtained with the physiological substrate were significantly different from that obtained with the synthetic substrate (Fig. 4). The differential inhibition of *p*-NPM-cleaving activity of the liver Golgi membranes is not due to lysosomal α-D-mannosidase, since PCMPS does not inhibit this activity [33]. These data suggest the possibility that the *p*-NPM and GlcNAcMan₅GlcNAc substrates are cleaved by different catalytic sites present on liver mannosidase II. Moreover, if the synthetic and physiological substrates are cleaved by different active sites, one would not expect them to be competitive inhibitors. Indeed, when the GlcNAcMan₅GlcNAc-cleaving activity of the liver Golgi membrane preparation was assayed in the presence of increasing amounts of *p*-NPM, the synthetic substrate had little or no effect

Fig. 4. Effect of PCMPS on rat mannosidases

Liver Golgi mannosidase II was assayed using 4 mM-*p*-NPM (○) or about 4000 c.p.m. of GlcNAcMan₅GlcNAc- (●). Epididymal mannosidase activity was assayed in the salt-washed Golgi membranes using about 4000 c.p.m. of GlcNAcMan₅GlcNAc- (△). The assays were carried out in 0.1 M-sodium acetate buffer, pH 6.0, as described in the Materials and methods section, except that the concentration of PCMPS in the reaction mixture was varied as shown. Under these conditions, > 15% of the GlcNAcMan₅GlcNAc-substrate was cleaved (100% activity) and released as free [³H]mannose (> 600 c.p.m.).

Table 4. Effect of metal ions, mercaptoethanol and PCMPS on rat epididymal mannosidase II-like enzyme

The stock reagent solution was added to the epididymal Golgi membrane solution in 0.1 M-sodium acetate buffer, pH 6.0. After the mixture had been kept on ice (0–4 °C) for 10 min, the reaction was started by adding substrate (about 4000 c.p.m. of GlcNAc[³H]-Man₅GlcNAc) and incubating at 37 °C for 2 h. The reaction was stopped by heat treatment, and the [³H]mannose released was separated from oligosaccharide on a column of Bio-Gel P-2 and quantified [2]. Without additions, the enzyme activities in liver and epididymal Golgi membranes hydrolysed nearly 18% of the substrate, or about 720 c.p.m. was released as free [³H]mannose.

Effectors	Concn. (mM)	Extent of hydrolysis (% of [³ H]mannose released)	Relative activity (%)
None	–	14.4	100
CuSO ₄	1	0.4	3
CoCl ₂	1	12.8	89
ZnCl ₂	1	10.8	75
MgCl ₂	1	12.3	85
MnCl ₂	1	11.5	80
EDTA	5	12.1	84
Mercaptoethanol	1	12.1	84
PCMPS	1	13.7	95

on the oligosaccharide-cleaving activity up to the highest concentration (15 mM) used (results not shown). These results are consistent with the possibility that the two substrates are recognized and hydrolysed by different catalytic domains.

In addition to Cu²⁺ (Table 4), swainsonine, an indolizidine alkaloid [34], was a potent inhibitor of the GlcNAcMan₅GlcNAc-cleaving activities of the liver and epididymal Golgi membranes and *p*-NPM-cleaving activity of liver mannosidase II (Fig. 5). At a concentration of 0.2 µM, the alkaloid caused over 50% inhibition. Nearly all of the enzyme was inactivated at a concentration of 5 µM and higher.

Fig. 5. Effect of swainsonine on rat mannosidases

Liver Golgi mannosidase II and epididymal mannosidase II-like enzyme were immunoprecipitated with IgG-sorb preincubated with anti-(mannosidase II) antibody. The washed antibody-antigen complex was suspended in 25 μ l of 100 mM-sodium acetate buffer, pH 6.0. The enzyme activities were assayed as described in Fig. 4, except that the concentration of swainsonine was varied as shown. Without additions, the enzyme activities from liver (*p*-NPM, $\circ$; GlcNAcMan₆GlcNAc-, $\bullet$) and epididymis (GlcNAcMan₆GlcNAc-, $\triangle$) hydrolysed >15% and 13% of the GlcNAcMan₆GlcNAc respectively. The antigen-antibody complex prepared from epididymal Golgi membranes showed no activity towards *p*-NPM.

Fig. 6. Immunoprecipitation of rat mannosidases with anti-(mannosidase II) antibody

Salt-washed Golgi membranes prepared from rat liver ($\bullet$) and epididymis ($\circ$) were solubilized by repeated extraction with detergent/salt solution. Portions from the solubilized membrane preparations were added to 100 μ l of IgG-sorb to which the indicated amounts of anti-(liver mannosidase II) antibody had been preadsorbed. This suspension was kept at room temperature for 60 min with occasional mixing, and then centrifuged in a microfuge for 2 min. The enzyme activity remaining in the supernatant was determined at pH 6.0 (0.1 M-sodium acetate buffer) with about 4000 c.p.m. of GlcNAcMan₆GlcNAc-. The solubilized liver and epididymal Golgi membranes incubated with IgG-sorb (without the antibody) released nearly 14% of the radioactivity (> 540 c.p.m.) as free [³H]mannose.

Immunoprecipitation studies

These studies show that the antibody to liver Golgi mannosidase II cross-reacted with both liver and epididymal enzyme activities (Fig. 6), indicating that the enzymes from the two tissues share a common immunogenic site(s) and/or the enzymes are derived from a common precursor.

Structural studies of the liver and epididymal enzymes

The cross-reactivity of the anti-(rat liver mannosidase) antibody with the liver and epididymal enzymes (see above) made it

Fig. 7. Immunodetection of liver and epididymis mannosidases by using anti-(rat liver mannosidase II) antibody

The rat liver and epididymis Golgi membranes were resolved on SDS/PAGE (a) or in the non-denaturing Davis system (b), transferred to nitrocellulose filters, and the bands were immunodetected with anti-(mannosidase II) antibody. Other details are described in the Materials and methods section. Lanes 1 and 3, epididymal Golgi membranes; lanes 2 and 4, liver Golgi membranes.

possible to investigate the structural relationship between the enzymes from these tissues. In the first set of experiments, we resolved purified Golgi membranes from these tissues by SDS/PAGE and detected the enzyme with anti-(mannosidase II) antibody. The results from these studies, presented in Fig. 7, show that, whereas the liver Golgi mannosidase II resolved as a doublet of M_r 120000 and 122000, only the M_r -120000 band was observed from the epididymis.

The rat liver mannosidase II has previously been shown to contain an intact subunit of M_r 124000 [15], a value similar to the M_r of the doublet reported above. In addition, the liver Golgi membranes showed the presence of two higher- M_r (160000–200000) immunoreactive bands. However, these two bands were shown to be biosynthetically unrelated to mannosidase II [15,35]. In the studies presented here, both liver and epididymal Golgi membranes showed the presence of these higher- M_r immunoreactive bands (Fig. 7). The resolution of liver mannosidase II as a doublet of M_r 122000 and 120000, instead of a single subunit of M_r 124000, is probably due to the high-resolution electrophoretic conditions used in the present study.

We also examined oligomeric form(s) of the enzyme from these tissues, using anti-(mannosidase II) antibody. The results in Fig. 7 show that, whereas the antibody cross-reacted with a major and several (two to four) minor forms from liver, only a major and perhaps a minor, immunoreactive band(s) was observed in the epididymis (Fig. 6). Furthermore, the major immunoreactive band in the two tissues shows different mobility, a result suggesting that the two tissues contain different charge and/or size isomers.

DISCUSSION

The data presented here show the occurrence of mannosidase II-like enzyme in the rat epididymis. This enzyme has a number of properties that differentiate it from the acid (lysosomal) α -D-mannosidase purified from this tissue [12]. First, unlike the lysosomal mannosidase, which hydrolyses synthetic substrate (*p*-NPM) at an optimal pH of 4.5, the mannosidase described in this report showed no activity towards this substrate (Fig. 1). The enzyme is active only towards oligosaccharide substrate (GlcNAcMan₆GlcNAc-) with a pH optimum of 6.0 (Fig. 1). Second, the enzyme described in this report is an integral Golgi membrane component and requires detergent for complete

solubilization (Table 1). Third, the enzyme solubilized from the epididymal particulate fraction (Table 1) or Golgi-rich fraction cross-reacted with anti-(rat liver Golgi mannosidase II) (Fig. 5), and not with the antibody made against homogeneous preparations of the epididymal (lysosomal) α -D-mannosidase (Table 1). The epididymal mannosidase is also different from rat liver endoplasmic α -D-mannosidase [29,30], cytosolic α -D-mannosidase [36] and Golgi mannosidase II [1,2], all of which are known to hydrolyse *p*-NPM efficiently.

The epididymal mannosidase is also different from the α 1,2-specific mannosidases purified from rat liver Golgi membranes [2,11,31], calf liver microsomes [37] and rabbit liver microsomes [38], because, unlike these mannosidases, the epididymal enzyme cleaves α 1,3- and α 1,6-linked mannosyl residues from GlcNAcMan₅GlcNAc- (Table 3). Moreover, the anti-(rat liver Golgi mannosidase IA) antibody showed no cross-reactivity with the GlcNAcMan₅GlcNAc-cleaving enzyme activity present in epididymal particulate fraction (Table 1). The epididymal enzyme is also different from rat [8] and human [13] sperm plasma membrane mannosidases, which show a rather broad substrate specificity cleaving α 1,2-, α 1,3- and α 1,6-linked mannosyl residues from a variety of substrates.

The epididymal mannosidase is remarkably similar to liver Golgi mannosidase II. The two enzymes show little or no activity towards substrates containing α 1,2-linked mannosyl residues (Table 3; also see ref. [2]). They are equally inhibited by swainsonine (Fig. 5), and they share a common antigenic site(s) (Figs. 6 and 7). This high degree of similarity suggests that the two enzymes are derived from a common precursor. The fact that the epididymal enzyme, like liver Golgi mannosidase II, cleaves α 1,3- and α 1,6-linked mannosyl residues from GlcNAcMan₅GlcNAc (Table 3) suggests that it is functionally similar to Golgi mannosidase II. Thus the inhibition of mannosidase II-like activity in the epididymal epithelial cells in the presence of swainsonine is probably the cause of the production of abnormally processed *N*-linked glycoproteins containing hybrid-type oligosaccharides instead of complex-type [9].

Despite numerous similarities, the epididymal Golgi mannosidase described in this report appears to be structurally different from the liver Golgi mannosidase II. Unlike liver mannosidase II, which hydrolyses both synthetic (*p*-NPM) and physiological (GlcNAcMan₅GlcNAc-) substrates, the epididymal enzyme cleaves only the physiological substrate. The lack of activity towards *p*-NPM appears to be the reason why we were unable to obtain evidence for the presence of this enzyme [5].

It is noteworthy that epididymal Golgi mannosidase II-like enzyme shows activity towards only natural substrate, whereas liver Golgi mannosidase II shows activity towards both synthetic and natural substrates [1,2]. Several lines of evidence suggest that the catalytic domain for the two substrates may be localized at different sites. (1) The differential inhibition of the *p*-NPM and GlcNAcMan₅GlcNAc-cleaving activities of liver mannosidase II by PCMPS (Fig. 4) is probably due to different sensitivity of the two sites to the inhibitor. (2) The lack of inhibition of GlcNAcMan₅GlcNAc-cleaving activity of liver Golgi mannosidase II by *p*-NPM is also consistent with the presence of two catalytic sites. (3) The liver and epididymal Golgi membranes resolved on SDS/PAGE show different immunoreactive subunits (Fig. 7a). Collectively, these results allow us to suggest that the catalytic domain for the oligosaccharide substrate may be localized in the subunit (*M*_r 120000) that is common to the liver and epididymis, and the catalytic domain for the *p*-NPM (in liver mannosidase II) may be localized in the *M*_r 122000 subunit (Fig. 7a). Indeed, immunodetection studies on oligomeric forms of the liver and epididymal Golgi enzymes showed several charge and/or size isomers in these tissues (Fig. 7b). Isolation of these

immunoreactive isomers for further chemical characterization will be needed to provide proof for the presence of two catalytic sites.

The Golgi apparatus plays a central role in the conversion of *N*-linked glycoproteins containing high-mannose oligosaccharides into hybrid-/complex-type structures [39]. This conversion requires the removal of α 1,2-linked mannosyl residues by Golgi mannosidases IA and IB [2,32], addition of a terminal *N*-acetylglucosamine by glucosaminyltransferase [40] and subsequent removal of α 1,3- and α 1,6-linked mannosyl residues by mannosidase II [2,40,41]. In addition, recent studies provide evidence suggesting the presence of an endomannosidase (glucosylmannosidase) in rat Golgi membranes [42,43] and rat brain microsomes [26]. The precise role of the endomannosidase in the processing and maturation of *N*-linked glycoproteins is not yet known. Although most of the processing enzymes have been chemically characterized from liver Golgi membranes, to the best of our knowledge the mannosidase II-like enzyme reported in this paper is the only processing enzyme identified in the rat epididymis. In view of the widespread implications of glycoproteins in sperm maturation (for reviews see refs. [44–47]), it would be of interest to identify and characterize chemically other processing enzymes of male reproductive tissues.

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