Isolation and Characterization of the Two Glycosylation Isoforms of Low Molecular Weight Mannose 6-Phosphate Receptor from Bovine Testis

EFFECT OF CARBOHYDRATE COMPONENTS ON LIGAND BINDING*

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Low molecular weight mannose 6-phosphate receptor from bovine testis exhibits two isoforms on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with M, values of 45,000 (MPR-2A) and 41,000 (MPR-2B), respectively. Each isoform was purified to near homogeneity by the sequential application of differential centrifugation and affinity chromatography. The isoforms contain a common polypeptide core, but differ in the carbohydrate content. Treatment with specific endoglycosidases demonstrated that each isoform contains two high mannose and/or hybrid and two complex N-linked oligosaccharide chains. The results obtained from treatment of each isoform with endo-β-galactosidase and neuraminidases and from lectin affinity chromatography reveal that MPR-SA contains a linear polylactosamine sequence(s) comprised of approximately 5 lactosamine units. A majority of the outer branches of the complex chains associated with MPR-2A are terminated with sialic acid residues. In contrast, MPR-2B lacks a polylactosamine sequence and a majority of the outer branches of the complex chains are terminated with galactose residues. MPR-2A exhibited a lower affinity than MPR-2B for mannose 6-phosphate-containing ligands. Treatment of MPR-2A with endo-β-galactosidase and neuraminidases followed by affinity chromatography revealed that polylactosamine and sialic acid residues impair the ability of MPR-2A to bind ligands.

The low molecular weight mannose 6-phosphate receptor (MPR-2) is one of two identified membrane-associated glycoproteins that bind ligands carrying covalently associated Man-6-P residues (1, 2). Each receptor has been implicated in the intracellular trafficking of acid hydrolases (3). The molecular weight of MPR-2 is variable depending on the tissue source. Receptor preparations isolated from bovine liver and a murine macrophage cell line (P388D1) have an apparent M, of 46,000 (4); those from human liver and human skin fibroblasts exhibit an apparent M, of 43,000 (5, 6). Gene cloning and sequencing studies indicate that bovine and human MPR-2 contains a polypeptide core of 28,000-29,000 consisting of an extracellular domain, a transmembrane domain, and an intracellular domain (7, 8). Bovine and human MPR-2 contains five potential N-glycosylation sites in its extracellular domain (7, 8). The results of in vitro translation studies with bovine MPR-2 suggest that four sites are glycosylated (7).

Early studies indicated that N-glycosylation of MPR-2 is essential for the acquisition of ligand binding ability (4, 9). However, a recent report suggests that N-glycosylation may not be required for the binding activity of the receptor, but may substantially increase the stability of the receptor (10).

This manuscript describes: 1) means for the isolation and purification of two MPR-2 glycosylation isoforms present in bovine testis, MPR-2A and MPR-2B; 2) partial characterization of the oligosaccharide chains carried by each isoform; and 3) the effect of the carbohydrate constituents associated with MPR-2 on its ability to bind ligand containing Man-6-P.

The results of preliminary studies have been reported (11).

MATERIALS AND METHODS

Frozen bovine and mouse testes, liver, and lung were purchased from Pel-Freeze Biologicals. Man-6-P, methyl-α-mannoside, methyl-α-galactoside, N-acetylneuraminic acid, and N-acetylglucosamine were obtained from Sigma; Endo H, Endo F, PNGase, and Endo-β-Galase, Genzyme; LFA, Arthrobacter ureafaciens and Vibrio cholerae neuraminidases, Calbiochem; Ham’s F-12 medium, Dulbecco’s modified Eagle’s medium, horse serum, and fetal bovine serum, Gibco-Bethesda Research Laboratories. Carrier-free Na[35]I and [35]S]methionine were obtained from Amersham. Mouse Leydig cells (T3M) and Sertoli cells (TM4) were obtained from the American Type Culture Collection. Immobilized lectins were obtained from the following sources: concanavalin A-agarose, WGA-Sepharose 6MB, UEA I-agarose, RCA I-agarose, PSA-agarose, and PHA-E-agarose, Sigma; DSA-Sepharose 4B, PWM-Sepharose 4B, LBA-Sepharose 4B, and Lotus A-Sepharose 4B, E-Y Laboratories. LFA-Affi-Gel 10 was prepared by coupling purified lectin to Affi-Gel 10 (Bio-Rad) according to the procedure recommended by the manufacturer (specific activity, 1.5 mg of protein/ml gel). Newcastle disease virus neuraminidase, N,N′-diacetylchitobiose, and Griffonia simplicifolia I isolectin-Sepharose 4B were generous gifts from Dr. I. Goldstein (the University of Michigan). A mixture of lysosomal enzymes from Dictyostelium discoideum was kindly provided by Dr. A. Kaplan (St. Louis Univer-

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Glycosylation Isoforms of Low Molecular Weight Man-6-P Receptor

Preparation of MPR-2 Glycosylation Isoforms

Unless otherwise indicated, all procedures were performed at 4 °C. Thawed bovine testes (400 g of decapsulated tissue) were cut into 1-cm cubes and homogenized in a Waring blender for 1 min in 1,200 ml of a buffer containing 10 mM EDTA, 10 mM 1,10-phenanthroline, and 20 mM imidazole, pH 7.0. The homogenate was centrifuged for 10 min at 800 × g. The supernatant fraction was adjusted to pH 5.0 by the addition of 2 N acetic acid, and the precipitated membranes were collected by centrifugation for 20 min at 10,000 × g. The pellets from each centrifugation step were extracted for 1 h with 20 g/ml of the above described buffer supplemented with 0.4 M KCl and 1.0% Triton X-100 (buffer A). The extracts were centrifuged for 1 h at 22,000 × g. The supernatants were adjusted to pH 6.5 and immediately applied to separate 2- × 6-cm affinity columns of agarose-(Man)-P. Each column was pre-equilibrated with a buffer comprised of 150 mM NaCl, 0.02% NaN3, 0.05% Triton X-100, 10 mM EDTA, 10 mM 1,10-phenanthroline, and 50 mM imidazole, pH 6.5 (buffer B). The columns were then washed with 1 liter of buffer B and eluted sequentially with increasing concentrations of Man-6-P (0.5 mM, 1.0 mM, and 2.0 mM, respectively, contained in 90 ml of buffer B). Fractions of 15 ml were collected. MPR-2 was detected with a radioimmunoassay procedure (14). Fractions enriched in MPR-2A or MPR-2B were pooled and diluted with 3 ml of equilibrating buffer. With the exception of PWM, each column was equilibrated with a buffer comprised of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% NaN3, and 0.1% Triton X-100. CaCl2 (1 mM) was included in samples containing V. cholerae, V. parahaemolyticus, and V. cholerae, and 50 mM sodium acetate buffer, pH 5.5, containing 0.05% Triton X-100. CaCl2 (1 mM) was included in samples containing V. cholerae, N. meningitidis, and V. cholerae.

Determination of N-terminal and Amino Acid Sequence

The N-terminal sequence of MPR-2A and MPR-2B was determined by the procedure of Hewick et al. (16), the amino acid composition by the procedure of Fujiwara et al. (17), and the neutral and amino sugar constituents by the procedure of Perini and Peters (18). Each analysis was kindly performed by Dr. F. Perini in the University of Michigan Protein Core Facility.

Treatment of 125I-MPR-2A and 125I-MPR-2B with Glycosidases

Endo H—125I-MPR-2A or 125I-MPR-2B (20,000 cpm) was boiled for 3 min in 30 µl of a solution containing 30 mM 2-mercaptoethanol and 0.5% SDS. The denatured receptors were mixed with 3 volumes of a buffer containing 0.02% bovine serum albumin, 0.1% Triton X-100, 100 mM sodium acetate, pH 5.8, and varying concentrations of Endo H (0–25 microunits/ml). The reaction mixtures were incubated at 37 °C for the times specified in the figure legends. The reactions were stopped by the addition of an equal volume of SDS-PAGE sample buffer and boiling for 3 min.

Endo β-Galase—The reaction mixtures (in total final volumes of 20 µl) contained 125I-MPR-2A or 125I-MPR-2B (10,000 cpm), buffer (50 mM sodium acetate, pH 5.8, 0.05% Triton X-100, 0.02% bovine serum albumin), and Endo β-Galase (0–200 microunits/ml). The reaction mixtures were incubated at 37 °C for the times specified.

Neuraminidase—The reaction mixtures (in final volumes of 20 µl) contained 125I-MPR-2A or 125I-MPR-2B (10,000 cpm), 0.1 unit/ml of neuraminidase (from A. ureafaciens, V. cholerae, and V. parahaemolyticus), and 50 mM sodium acetate buffer, pH 5.5, containing 0.05% Triton X-100. CaCl2 (1 mM) was included in samples containing V. cholerae, N. meningitidis, and V. cholerae.

Partial Digestion of 125I-MPR-2A and 125I-MPR-2B with Proteinases

125I-MPR-2A or 125I-MPR-2B (80,000 cpm) was deglycosylated exhaustively with PNGase (10 units/ml for 20 h) as described above. The deglycosylated preparations were diluted with 1 volume of 50 mM Tris-HCl buffer, pH 7.8, and incubated at 37 °C for 45 min with 2.5 µg/ml trypsin or chymotrypsin. The resulting reaction mixtures were boiled with an equal volume of SDS-PAGE sample buffer and subjected to SDS-PAGE.

Mild Acid Hydrolysis of 125I-MPR-2A and 125I-MPR-2B

125I-MPR-2A or 125I-MPR-2B (20,000 cpm) contained in 100 µl of 0.1 N HCl was incubated at 80 °C for 50 min. The hydrolysates were transferred to Centricon-30 concentrators (Amicon). HCl was removed by addition of 1 ml of a solution comprised of 150 mM NaCl and 0.05% Triton X-100 followed by centrifugation at 3,000 × g; this procedure was repeated once. The retentate (40 µl) was boiled in SDS-PAGE sample buffer for 3 min.

Chromatography of 125I-MPR-2A and 125I-MPR-2B on Immobilized Lectin Columns

Immobilized lectins (0.4 ml each) were packed in 0.7- × 4-cm columns. With the exception of immobilized PSA, each column was equilibrated with a buffer comprised of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% NaN3, and 0.1% Triton X-100. Columns containing immobilized PSA were equilibrated in the same buffer containing 1 mM MgCl2 and 1 mM CaCl2. The columns were equilibrated and eluted at room temperature. Enzyme-treated and untreated receptor preparations (approximately 20,000 cpm) contained in 100 µl of appropriate buffer and run into each column. The column flow was stopped to allow interaction of the receptor isoforms with each lectin. After 15 min, the columns were opened and washed with 3 ml of equilibrating buffer. With the exception of PWM, the columns were eluted with 3 ml of equilibrating buffer containing an appropriate monosaccharide or monosaccharide derivative (Table I). The PWM-Sepharose 4B columns were eluted with 3 ml of a buffer containing an addition of an equal volume of SDS-PAGE sample buffer and boiling for 3 min.

TABLE I

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Monosaccharides/monosaccharide derivatives used for elution of receptor isoforms from immobilized lectins</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono- and disaccharides</td>
<td>mm</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>Methyl-α-mannoside</td>
<td>1250</td>
</tr>
<tr>
<td>DSA</td>
<td>N,N′-Diacetylchitobiose</td>
<td>25</td>
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<tr>
<td>GS F</td>
<td>Methyl-α-galactoside</td>
<td>50</td>
</tr>
<tr>
<td>LBA</td>
<td>N-Acetylgalactosamine</td>
<td>50</td>
</tr>
<tr>
<td>LFA</td>
<td>N-Acetylmuramic acid</td>
<td>10</td>
</tr>
<tr>
<td>Lotus A</td>
<td>L-Fucose</td>
<td>50</td>
</tr>
<tr>
<td>PHA-E</td>
<td>N-Acetylgalactosamine</td>
<td>600</td>
</tr>
<tr>
<td>PSA</td>
<td>Methyl-α-mannoside</td>
<td>300</td>
</tr>
<tr>
<td>RCA I</td>
<td>Lactose</td>
<td>300</td>
</tr>
<tr>
<td>UEA I</td>
<td>L-Fucose</td>
<td>300</td>
</tr>
<tr>
<td>WGA</td>
<td>N-Acetylgalactosamine</td>
<td>300</td>
</tr>
</tbody>
</table>

a Unless indicated otherwise, all monosaccharides and monosaccharide derivatives were of β configuration.

b G. simplificioides 1 isolecitin.
Isolation of 2 MPR-2 Glycosylation Isoforms of Bovine Testis

The isolation of MPR-2 from bovine testis has been described previously (14). The present manuscript describes the isolation of two MPR-2 glycosylation isoforms with apparent \( M_r \) values of 45,000 (MPR-2A) and 41,000 (MPR-2B), respectively (Fig. 1A). While variable from preparation to preparation, the average ratio of MPR-2A to MPR-2B, as assessed by densitometric scanning of gels stained with Coomassie Blue, approximated 1:4.

Partial Resolution of MPR-2A and MPR-2B by Differential Centrifugation—Bovine testis homogenates were centrifuged for 10 min at 800 \( \times \) \( g \). The approximate ratio of MPR-2A to MPR-2B in the 800 \( \times \) \( g \) pellet was 3:2 (Fig. 1B, lane 1) as compared to a ratio of 1:4 found in the total homogenate (Fig. 1A). Membranes contained in the 800 \( \times \) \( g \) supernatant fraction were precipitated by adjusting the suspension to pH 5 with acetic acid. The precipitated membrane fraction was nearly devoid of MPR-2A and contained only small amounts of MPR-1 (Fig. 1B, lane 2).

Isolation of MPR-2A—The detergent extract from the 800 \( \times \) \( g \) pellet (enriched in MPR-2A) was applied to an agarose-(Man)_5-P affinity column. The receptor isoforms were differentially eluted from the column with increasing concentrations of Man-6-P (Fig. 2A). The 0.5 mM Man-6-P eluate was highly enriched in MPR-2A (Fig. 2A, SDS-PAGE, lane a). As the concentration of Man-6-P was increased, the amount of MPR-2A present in the eluate decreased with a concomitant increase in MPR-2B. Fractions enriched with MPR-2A were pooled (Fig. 2A, pool a), dialyzed, and again applied to, and eluted from, a 1.0- \( \times \) 4.0-cm agarose-(Man)_5-P affinity column. The 0.5 mM Man-6-P eluate contained nearly homogeneous MPR-2A and lacked detectable MPR-1 (Fig. 2B, SDS-PAGE, lane a').

Isolation of MPR-2B—The membrane fraction prepared by adjusting the 800 \( \times \) \( g \) supernatant to pH 5.0 was extracted with Triton X-100 and subjected to affinity chromatography. The column was first eluted with 0.5 mM Man-6-P to remove traces of MPR-2A and then with 2.0 mM Man-6-P to elute MPR-2B (data not shown). MPR-1 present in the 2 mM Man-6-P eluate was removed by affinity chromatography on a column containing a mixture of lysosomal enzymes obtained from D. discoideum immobilized on Affi-Gel 10 (4).

Approximately 50 \( \mu \)g of homogeneous MPR-2A and 500 \( \mu \)g of homogeneous MPR-2B were isolated from 400 g of decapsulated bovine testes.

Fig. 1. Partial resolution of MPR-2A and MPR-2B contained in membrane preparations of bovine testis homogenates by differential centrifugation. Panel A, a mixture of MPR-1 and MPR-2 was purified as previously described (14). The membrane fraction was precipitated by adjusting the crude bovine testis homogenate to pH 5.0. The resulting precipitate was centrifuged for 20 min at 10,000 \( \times \) \( g \). The membranes were extracted with 1% Triton X-100 and applied to an agarose-(Man)_5-P affinity column. The column was washed, and the receptors were eluted with 5 mM Man-6-P. Suitable aliquots were submitted to SDS-PAGE (10% gel). Panel B, crude bovine testis homogenate was resolved into a pellet and a supernatant fraction by centrifugation at 800 \( \times \) \( g \) for 10 min. Membranes from the supernatant fraction were precipitated by adjusting the pH to 5 with acetic acid. Each fraction was extracted with Triton X-100, and the receptors were purified and submitted to SDS-PAGE as described above. Lane 1, receptors extracted from 800 \( \times \) \( g \) pellet; lane 2, receptors extracted from the membranes present in the 800 \( \times \) \( g \) supernatant.

MPR-2A and MPR-2B Contain a Common Polypeptide Core

\(^{125}\)I-Labeled MPR-2A and MPR-2B were treated with PNGase to remove N-linked oligosaccharides (29) and submitted to SDS-PAGE. Each deglycosylated isoform migrated with an apparent \( M_r \) of 28,700, as deduced from its cDNA sequence (7), the 29,000 band is believed to be a completely deglycosylated form of MPR-2A and MPR-2B. Based on the calculated \( M_r \) values before, and after, deglycosylation, the carbohydrate content of MPR-2A and MPR-2B approximated 36% and 29%, respectively. These values are in good agreement with those values determined by chemical analysis (Table II). Trace amounts of bands with \( M_r \) values of 42,000 and 26,000 were also detected in \(^{125}\)I-MPR-2A preparations before, and after, deglycosylation. These bands are believed to be proteolytic products arising from the \( M_r \) = 45,000 and 29,000 bands. When deglycosylated \(^{125}\)I-MPR-2A and \(^{125}\)I-MPR-2B were partially digested with trypsin, or chymotrypsin, and analyzed on SDS-PAGE, nearly identical proteolytic profiles were observed (Fig. 3B). Each receptor isoform contained the same N-terminal sequence, Thr-Glu-Glu-Lys—. Additionally, the amino acid composition of each isoform was nearly identical (Table II). Taken together, the above results suggest that MPR-2A and MPR-2B contain a common polypeptide core, but differ in carbohydrate content.

MPR-2A and MPR-2B Are Different in Polylactosamine and Sialic Acid Content

MPR-2A and MPR-2B were examined for the presence of polylactosamine sequences. The \(^{125}\)I-labeled isoforms were treated with Endo-\( \beta \)-Galase, an enzyme known to cleave polylactosamine sequences (21). The \( M_r \) of MPR-2A, but not MPR-2B, was reduced approximately by 2,000 by the action of Endo-\( \beta \)-Galase (Fig. 4). These results suggest that MPR-
FIG. 2. Separation of MPR-2A from MPR-2B by affinity chromatography. The 800 × g pellet (Fig. 1B, lane 1) was extracted with Triton X-100 and subjected to affinity chromatography as described under "Materials and Methods." Panel A, the affinity column was eluted with increasing concentrations of Man-6-P as indicated. The presence of MPR-2 was detected by a radioimmunoassay procedure (14). Fractions (a–e) were arbitrarily pooled as indicated and subjected to SDS-PAGE (10% gel). Panel B, fraction a (see panel A) was dialyzed and again subjected to affinity chromatography in the manner described for panel A. Pooled fractions (a′–e′) were submitted to SDS-PAGE.

FIG. 3. Comparison of polypeptide cores of MPR-2A and MPR-2B. Panel A, 125I-MPR-2A (lanes 1 and 3) and 125I-MPR-2B (lanes 2 and 4) were incubated in the presence, or absence, of PNGase (10 units/ml) as described under "Materials and Methods." The resulting reaction mixtures (∼4,000 cpm) were submitted to SDS-PAGE (10% gel). Panel B, deglycosylated 125I-MPR-2A (lanes 1 and 3) and 125I-MPR-2B (lanes 2 and 4) were treated with chymotrypsin or trypsin as described under "Materials and Methods." Each reaction mixture was submitted to SDS-PAGE (17% gel).

MPR-2A contains approximately 5 N-acetyllactosamine repeat units.

The contribution of sialic acid residues to the M₉ of each isoform was also examined. 125I-MPR-2A was treated separately with A. ureafaciens, V. cholerae, or C. perfringens neuraminidase. In each instance, the M₉ was reduced by 1,500 to 2,000. In contrast, the M₉ of 125I-MPR-2B was not altered by treatment with any of the three neuraminidases (Fig. 5A). Similar results were obtained when each isoform was subjected to mild acid hydrolysis (Fig. 5B). These results suggest that MPR-2A, but not MPR-2B, is highly sialylated (5–7 sialic acid residues/receptor molecule).

MPR-2A and MPR-2B Each Contain Two High Mannose/Hybrid and Two Complex N-Linked Oligosaccharides

125I-MPR-2A and 125I-MPR-2B were each digested with endoglycosidases and submitted to SDS-PAGE. The number and types of N-linked oligosaccharides present on each isoform were determined by examining the change in mobility and the number of 125I-labeled bands on SDS-PAGE gels before, and after, glycosidase treatment.

High Mannose and/or Hybrid Oligosaccharides—When 125I-MPR-2B was incubated with increasing concentrations of Endo H (2–25 milliunits/ml at 37 °C for 1 h), 3 distinct bands of 41,000, 39,000, and 37,000, respectively, were detected (Fig. 6B). Exhaustive treatment of this isoform with Endo H (25 milliunits/ml for 20 h) generated 2 bands, a major band of 37,000 and a minor band of 39,000. These results indicate that the majority of the MPR-2B molecules contained 2 high mannose or hybrid type chains, each with an apparent M₉ approximating 2,000. A small portion of MPR-2B molecules contained only 1 high mannose or hybrid chain.

The apparent M₉ of 125I-MPR-2A was reduced from 45,000 to 41,000 when the receptor was treated exhaustively with Endo H (Fig. 6A). However, when 125I-MPR-2A was partially digested with Endo H, the digestion products migrated as a single diffuse band. This observation is attributed to the heterogeneity of the polylactosamine sequence(s) present in MPR-2A since when 125I-MPR-2A was preincubated with Endo-β-Galase prior to the incubation with Endo H, 3 distinct bands with apparent M₉ values of 43,000, 41,000, and 39,000,
TABLE II

Amino acid and carbohydrate composition of MPR-2A and MPR-2B

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MPR-2A</th>
<th>MPR-2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol % of total aa*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>12.3</td>
<td>12.3</td>
</tr>
<tr>
<td>Thr</td>
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<td>Ser</td>
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<td>Leu</td>
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<table>
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<tr>
<th>Carbohydrate</th>
<th>MPR-2A</th>
<th>MPR-2B</th>
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<tbody>
<tr>
<td>nmol/mg of total AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>909</td>
<td>589</td>
</tr>
<tr>
<td>GlcN</td>
<td>1052</td>
<td>763</td>
</tr>
<tr>
<td>Gal</td>
<td>617</td>
<td>353</td>
</tr>
<tr>
<td>Fuc</td>
<td>66</td>
<td>47</td>
</tr>
</tbody>
</table>

*aa, amino acid residues.
| Glu includes Glu and Gln. |
| Arg includes Asp and Asn. |
| ND, not determined. |

The total carbohydrate by weight (not including sialic acid) was 34% and 26% of the total glycoprotein for MPR-2A and MPR-2B, respectively.

Fig. 4. Treatment of 125I-MPR-2A and 125I-MPR-2B with Endo-β-Galase. 125I-MPR-2A (panel A) and 125I-MPR-2B (panel B) (10,000 cpm) were incubated separately at 37 °C for 2 h in the presence of 0.1 unit/ml of A. ureafaciens (lanes 2 and 6), V. cholerae (lanes 3 and 7), or C. perfringens neuraminidase (lanes 4 and 8) as described under "Materials and Methods." Aliquots of each reaction mixture were submitted to SDS-PAGE. Panel A, 125I-MPR-2A and 125I-MPR-2B (10,000 cpm) were incubated at 37 °C for 2 h in the absence (lanes 1 and 5) or presence, of 0.1 unit/ml of A. ureafaciens (lanes 2 and 6), V. cholerae (lanes 3 and 7), or C. perfringens neuraminidase (lanes 4 and 8).

Fig. 5. Effect of treatment of 125I-MPR-2A and 125I-MPR-2B with neuraminidase or mild acid hydrolysis. Panel A, 125I-MPR-2A and 125I-MPR-2B (10,000 cpm) were incubated at 37 °C for 2 h in the absence (lanes 1 and 5), or presence, of 0.1 unit/ml of A. ureafaciens (lanes 2 and 6), V. cholerae (lanes 3 and 7), or C. perfringens neuraminidase (lanes 4 and 8) as described under "Materials and Methods." Aliquots of each reaction mixture were submitted to SDS-PAGE. Panel B, 125I-MPR-2A and 125I-MPR-2B (20,000 cpm) were treated with 0.1 unit/ml of neuraminidase or 0.1 unit/ml of neuraminidase with varying amounts of Endo H as described under "Materials and Methods." Aliquots of each reaction mixture were submitted to SDS-PAGE. Lane 1 contained untreated 125I-MPR-2A, lane 2, untreated 125I-MPR-2B.

Fig. 6. Treatment of 125I-MPR-2A and 125I-MPR-2B with Endo H. Panel A, 125I-MPR-2A (10,000 cpm) was incubated at 37 °C for 2 h in the presence, or absence, of Endo-β-Galase (100 units/ml). Each sample was boiled for 3 min and then incubated at 37 °C with varying amounts of Endo H as described under "Materials and Methods." Panel B, 125I-MPR-2B was treated with Endo H as described in panel A without pretreatment with Endo-β-Galase. The samples were submitted to SDS-PAGE (10%).

respectively, were obtained. These results suggest that MPR-2A contains 2 high mannose or hybrid N-linked oligosaccharide chains, each with a M, approximating 2,000. The polylactosamine sequence(s) are not present on Endo H-sensitive chains since the observed change in the asparagine sequence(s) are believed to reside on a single complex chain. After exhaustive treatment with Endo H and PNGase yielded 3 bands, a major band of 5,000, a minor band of 4,000, and those associated with MPR-2A 5,000 and 7,000, respectively. The polylactosamine sequence(s) are believed to reside on a single complex chain since treatment of MPR-2A with Endo-β-Galase reduced the size of only one complex chain (Fig. 7A).

N-Linked Complex Oligosaccharides—125I-MPR-2A and 125I-MPR-2B were treated exhaustively with Endo H and then partially digested with PNGase. 125I-MPR-2B treated in this manner yielded 3 bands on SDS-PAGE with apparent M, values of 37,000, 33,000, and 29,000, respectively (Fig. 7B). 125I-MPR-2A treated in the same manner yielded 3 bands of 41,000, 36,000, and 29,000, respectively (Fig. 7A). Pretreatment of 125I-MPR-2A with Endo-β-Galase, followed by sequential digestion with Endo H and PNGase yielded 3 bands, 1 band with an apparent M, of 29,000 and 2 new bands with apparent M, values of 39,000 and 34,000 (Fig. 7A). These results suggest that each isofrom contains two complex chains. The apparent M, of the two complex oligosaccharides associated with MPR-2B approximated 4,000, and those associated with MPR-2A 5,000 and 7,000, respectively. The polylactosamine sequence(s) are believed to reside on a single complex chain since treatment of MPR-2A with Endo-β-Galase reduced the size of only one complex chain (Fig. 7A).

When 125I-MPR-2A was treated with Endo F and subjected to SDS-PAGE, a single band with an apparent M, of 41,000 was detected. In contrast, when 125I-MPR-2B was treated in the same manner, 2 bands were detected, a major band of 37,000 and a minor band of 39,000 (Fig. 8A). After exhaustive treatment with Endo H, neither 125I-MPR-2A nor 125I-MPR-2B was sensitive to Endo F (Fig. 8B). Since Endo F cleaves biantennary but not triantennary or tetra-antennary complex chains (20), these results suggest that the N-linked complex

Amino acid and carbohydrate composition of MPR-2A and MPR-2B

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MPR-2A</th>
<th>MPR-2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol % of total aa*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>12.3</td>
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</tr>
<tr>
<td>Thr</td>
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<tr>
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<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>MPR-2A</th>
<th>MPR-2B</th>
</tr>
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<tbody>
<tr>
<td>nmol/mg of total AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>909</td>
<td>589</td>
</tr>
<tr>
<td>GlcN</td>
<td>1052</td>
<td>763</td>
</tr>
<tr>
<td>Gal</td>
<td>617</td>
<td>353</td>
</tr>
<tr>
<td>Fuc</td>
<td>66</td>
<td>47</td>
</tr>
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</table>

*aa, amino acid residues.
| Glu includes Glu and Gln. |
| Arg includes Asp and Asn. |
| ND, not determined. |

The total carbohydrate by weight (not including sialic acid) was 34% and 26% of the total glycoprotein for MPR-2A and MPR-2B, respectively.
Fig. 7. Treatment of $^{125}$I-MPR-2A and $^{125}$I-MPR-2B with PNGase. Panel A, $^{125}$I-MPR-2A (10,000 cpm) was incubated in the presence, or absence, of Endo-β-Galase as described in Fig. 6. Each sample was then boiled for 3 min. Endo H (25 unit/ml) was added to each tube, and the reaction mixtures were incubated at 37°C. After 20 h, the reactions were stopped by boiling. Suitable aliquots of each reaction mixture were pipetted into tubes. Phosphate buffer, pH 8.6, was added to each tube at a final concentration of 100 mM. PNGase was added at the final concentrations indicated in the figure. The samples were incubated at 37°C for 20 h. The reactions were stopped by boiling with SDS-PAGE sample buffer. Suitable aliquots were submitted to SDS-PAGE (10%). Panel B, $^{125}$I-MPR-2B (10,000 cpm) was treated with Endo H and then PNGase as described in panel A without pretreatment with Endo-β-Galase.

Fig. 8. Treatment of $^{125}$I-MPR-2A and $^{125}$I-MPR-2B with Endo F. Panel A, $^{125}$I-MPR-2A and $^{125}$I-MPR-2B were incubated at 37°C for 20 h in the presence, or absence, of Endo F (24 unit/ml) as described under "Materials and Methods." The reaction mixtures were boiled and then Endo F (24 unit/ml) was added to the reaction mixtures as specified in the figure. The reaction mixtures were incubated at 37°C for 20 h and submitted to SDS-PAGE (10% gel).

Partial Characterization of the Structure of the N-Linked Oligosaccharides of MPR-2A and MPR-2B Using Immobilized Lectins

Binding to Immobilized DSA and PWM—The results obtained with immobilized DSA are presented in Fig. 9A. Thirty-two percent of $^{125}$I-MPR-2B and 66% of $^{125}$I-MPR-2A were bound to the immobilized DSA. The bound fraction of $^{125}$I-MPR-2A was reduced by approximately 50% after treatment of the isoform with Endo-β-Galase. DSA is known to bind unbranched (22, 23) and branched polylactosamine sequences (24), and oligosaccharides containing an outer α-mannose residue substituted with Galβ1,4GlcNAc at both C-2 and C-6 positions (23, 25, 26). The finding that treatment of MPR-2A with Endo-β-Galase partially abolished its ability to bind DSA suggests that each of the above described carbohydrate structures (i.e., polylactosamine sequence(s) and α-mannose residues substituted with Galβ1,4GlcNAc at C-2 and C-6 positions) are present on MPR-2A. While MPR-2B lacks a polylactosamine sequence, 32% of the isoform was bound to DSA (Fig. 9A), a result that suggests that MPR-2B carries N-linked oligosaccharides containing 2,6-substituted α-mannose residues. Neither MPR-2A nor MPR-2B was bound to immobilized PWM (Fig. 9B), a lectin that binds branched, but not unbranched, polylactosamine sequences (27), suggesting that the polylactosamine sequence(s) associated with MPR-2A are present in a linear array.

Binding to Immobilized LFA and WGA—$^{125}$I-MPR-2A exhibited a higher affinity for immobilized LFA (60% bound) than $^{125}$I-MPR-2B (17% bound) (Table III). LFA is known to bind oligosaccharides containing sialic acid linked via α2,3- and α2,6-glycosidic bonds (28). Binding of $^{125}$I-MPR-2A and...
perfingens terminated with galactose residues and those of MPR-2.4 with dase treatment. These results suggest that the majority of the simplicifolia sialic acid residues. They further suggest that the terminal respectively, after removal of sialic acid from the isoforms with C.

The binding of 125I-MPR-2A and 125I-MPR-2B was bound to immobilized concanavalin A. Only 6% of 125I-MPR-2A and 23% of 125I-MPR-2B were bound to immobilized PSA. Concanavalin A and PSA each bind high mannose type, hybrid, and biantennary complex oligosaccharides (35). However, oligosaccharides that bind PSA must be fucosylated on the core GlcNAc residues. The low order of binding of either of the receptor isoforms to PSA implies that only a small portion of the high mannose and/or hybrid N-linked oligosaccharides is fucosylated. 125I-MPR-2A and 125I-MPR-2B, 18% and 39%, respectively, were bound to immobilized PHA-E after treatment with neuraminidase. PHA-E binds oligosaccharides containing bisected structures (36). It is suggestive, therefore, that a portion of each isoform contains bisected N-linked oligosaccharides.

**Polygalactosamine and Sialic Acid Residues Impair the Binding of MPR-2A to Agarose-(Man)₅-P**

MPR-2A was eluted from the agarose-(Man)₅-P affinity column at relatively low concentrations of Man-6-P compared to MPR-2B, suggesting that MPR-2A has a lower affinity for Man-6-P than MPR-2B (Fig. 2). Since the isoforms differ in their polylactosamine and sialic acid content, the potential effects of each of these components on ligand binding were investigated. MPR-2A was incubated in the presence, or absence, of neuraminidase and/or Endo-β-Galase and subjected to affinity chromatography on agarose-(Man)₅-P. Treatment of MPR-2A with either neuraminidase or Endo-β-Galase enhanced binding of the receptor to the affinity matrix (Fig. 10). Neuraminidase treatment enhanced ligand binding to a greater extent than Endo-β-Galase treatment. When MPR-2A was treated with both glycosidases, the receptor exhibited a higher affinity for ligand than when treated with either enzyme alone. Indeed, the elution profile after treatment with both enzymes closely approximated that observed for MPR-2B. Taken together, these results suggest that the polylactosamine and sialic acid residues present on the complex chains of MPR-2A exert an additive inhibitory effect on its ligand binding affinity.

**MPR-2 Preparations from Different Bovine and Mouse Tissues and Cells Have Differing Sensitivity to Endo-β-Galase**

MPR-2 was isolated from several bovine and mouse tissues and submitted to SDS-PAGE. Distinct differences in the migration patterns were observed (Fig. 11A). Bovine liver MPR-2 contained 2 bands, a major band with apparent M₉ of 43,000 and a minor band of 40,000. MPR-2 preparations obtained from bovine kidney and lung migrated as diffuse bands, each with an apparent M₉ of 45,000. After incubation with Endo-β-Galase, a small decrease in M₉ (1,000–2,000) was detected in receptor preparations from kidney and lung, suggesting the presence of polylactosamine sequences (Fig. 11B). In contrast, the M₉ of liver MPR-2 remained unchanged. Mouse testis MPR-2 contained 2 bands with apparent M₉ values of 41,000 and 45,000 (Fig. 11A), values similar to those found for the bovine testis MPR-2 isoforms. Mouse liver MPR-2 migrated as single band with an apparent M₉ of 43,000, the same M₉ as that noted for the major band of bovine liver MPR-2.
Glycosylation Isoforms of Low Molecular Weight Man-6-P Receptor

FIG. 10. The effect of Endo-β-Galase and/or neuraminidase treatment on the binding of MPR-2A to an agarose-(Man)_6-P affinity column. Purified MPR-2A (15 µg) were incubated at 37 °C for 4 h with 0.2 unit/ml of Endo-β-Galase, 0.5 unit/ml of C. perfringens neuraminidase, or a mixture containing each enzyme. The enzyme-treated MPR-2A and controls (MPR-2A and MPR-2B incubated under the same conditions without enzyme) were applied to 0.7 × 1.0-cm agarose-(Man)_6-P columns pre-equilibrated at 4 °C with a buffer comprised of 50 mM imidazole, pH 6.5, 150 mM NaCl, 0.02% NaN₃, and 0.05% Triton X-100. The columns were washed with 1.8 ml of the buffer and then eluted with 1.8 ml of the same buffer containing increasing concentrations of Man-6-P as indicated in the figure. Fractions of 0.3 ml were collected. ○-○, untreated MPR-2A; □-□, untreated MPR-2B; ○-○, MPR-2A treated with Endo-β-Galase; □-□, MPR-2A treated with neuraminidase; and Δ-Δ-Δ, MPR-2A treated with both enzymes.

To address the question whether the receptor isoforms were derived from single or multiple types of testis cells, mouse Sertoli (TM4) and Leydig (TM3) cells were metabolically labeled with [³⁵S]methionine, and their MPRs were isolated by affinity chromatography. Receptor preparations from either cell type when subjected to SDS-PAGE each yielded a single band; the apparent Mᵣ for Sertoli cell MPR-2 was 45,000 and that for Leydig cell MPR-2, 43,000 (Fig. 12). Leydig cell MPR-2 was insensitive to the action of either Endo-β-Galase or neuraminidase. However, the Mᵣ of Sertoli cell MPR-2 was decreased by ~3,000 after treatment with Endo-β-Galase; treatment with neuraminidase did not alter its Mᵣ.

DISCUSSION

Bovine testis MPR-2 was shown to consist of a mixture of two glycosylation isoforms. The findings that each isoform had a polypeptide core with a Mᵣ of 29,000, that the amino acid composition of each isoform was nearly identical, that each polypeptide core had the same N-terminal amino acid sequence, and that the SDS-PAGE profiles of tryptic and chymotryptic digests of each isoform were nearly identical indicate that the isoforms share a common polypeptide core. The Mᵣ of the polypeptide core and the determined N-terminal sequence are in good agreement with those deduced from cDNA sequencing data (7).

The observed differences in the Mᵣ of the isoforms are attributed to differences in carbohydrate content. MPR-2A and MPR-2B contained 36% and 29% carbohydrate by weight, respectively, as determined by their differences in migration on SDS-PAGE before, and after, treatment with PNGase. Studies utilizing Endo-β-Galase and specific lectins.
revealed the presence of a polylactosamine sequence(s) on the N-linked complex oligosaccharides of bovine testis MPR-2A, but not those of MPR-2B. We suggest that the polylactosamine sequence(s) is unbranched since the isoform failed to bind to PWM, a lectin known to bind branched polylactosamine sequences (27). It seems unlikely that either isoform contains type A, B, or H antigens, antigens frequently associated with polylactosamine sequences (42), since MPR-2A and MPR-2B failed to bind to LbA, G. simplicifolia I isolectin, and UEA. MPR-2A is highly sialylated compared to MPR-2B. This was demonstrated by: 1) a decrease in the apparent Mr of MPR-2A, but not MPR-2B, after treatment with neuraminidase or after mild acid hydrolysis and 2) a higher affinity of MPR-2A for immobilized LFA, a sialic acid binding lectin, than MPR-2B.

The results of cDNA sequence studies by Dahms et al. (7) suggest that bovine MPR-2 contains 5 potential glycosylation sites. The present studies indicated that 4 of the 5 potential glycosylation sites present on each isoform are occupied. In MPR-2A and the majority of MPR-2B molecules, 2 of the 4 N-linked oligosaccharides are of the high mannose and/or hybrid types and 2 are processed to complex chains. A small portion of MPR-2B contains three complex chains. A similar finding was reported for MPR-2 isolated from BHK cells transfected with the human receptor gene (10). The apparent Mr values found for the complex chains of each isoform (4,000–7,000) and the finding that the complex chains were insensitive to Endo F indicate that the oligosaccharides are highly branched.

Although polylactosamine sequence(s) are present on MPR-2A, but not MPR-2B, the ratios of Man to GlcN and Man to Gal are similar for each isoform (Table II). This is attributed partly to the fact that a portion of MPR-2B contains one more complex chain than MPR-2A. Additionally, it is possible that MPR-2B contains more hybrid type chains than MPR-2A.

When bovine testis homogenates were subjected to differential centrifugation, a major portion of MPR-2A was found in the 800 x g pellet. This finding could be attributable to the segregation of MPR-2A and MPR-2B into separate subcellular organelles in a single cell type, or, alternatively, MPR-2A and MPR-2B may be present in distinct cell types that differ in their ability to resist mechanical rupture. The latter possibility is considered more likely since: 1) MPR-2 isolated from bovine and mouse tissues, other than testis, did not contain two distinct receptor isoforms; 2) MPR-2 preparations from cultured mouse Leydig and Sertoli cells each yielded a single band on SDS-PAGE and exhibited differing sensitivity to Endo-β-Galase; and 3) O'Brien et al. (37) demonstrated that MPR-2 from mouse pachytene spermatocytes and round spermatids each gave a single band on SDS-PAGE.

Polylactosamine sequences have been found in glycoproteins of diverse origin, including band 3 of human erythrocyte membranes (38), Chinese hamster ovary cells (39), GM-979 cells (40), and Ehrlich ascites tumor cells (41). The nature and content of the polylactosamine sequences vary widely during development and oncogenesis (42). The present finding that a polylactosamine sequence is present on only one of the two MPR-2 isoforms is of interest. Since MPR-2 is readily isolated and its gene has been cloned, the receptor should serve as a convenient acceptor for studying the mechanism of synthesis of polylactosamine sequences in vivo.

Diverse cell types exhibit variable levels of N-acetylgalcosaminyltransferase activities required for the synthesis of polylactosamine sequences (43–45). The presence of a polylactosamine sequence(s) on MPR-2A, but not MPR-2B, may be attributable to differences in N-acetylgalcosaminyltransferase activities present in different cell types of testis. In agreement with this contention is the observation that MPR-2 prepared from different bovine and mouse tissues and cells exhibited differing sensitivity to Endo-β-Galase. Other factors which may influence the expression of polylactosamine structures include the amino acid sequences of polypeptide cores (42) and the core structures of oligosaccharides with which polylactosamine sequences are associated, i.e. polylactosamine sequences are preferentially added to the C-6 branch of 2,6-substituted α-mannose residues (25, 26, 42). Since MPR-2A and MPR-2B contain the same polypeptide core, the former mechanism cannot be responsible for the observed differences. It is not yet possible to determine with certainty whether variations in the processing of N-linked oligosaccharides contribute to the presence of a polylactosamine sequence(s) on MPR-2A, but not on MPR-2B, since the complete structures of the complex N-linked oligosaccharide chains associated with each isoform are as yet undefined. However, present evidence suggests that the two isoforms contain comparable levels of α,2,6-substituted mannose residues since after treatment with Endo-β-Galase approximately one-third of each isoform was bound to DSA, a lectin known to bind oligosaccharides containing an outer mannose residue substituted at C-2 and C-6 with Galβ1,4GlcNAc (26).

The degree of sialylation of oligosaccharides depends on the activities of sialyltransferases present in individual cell types (46). The observed difference in sialic acid content of MPR-2A and MPR-2B may be due to different sialyltransferase activities in different cell types present in bovine testis.

Of considerable interest is the observation that the polylactosamine sequence(s) and sialic acid residues of MPR-2A impair ligand binding. The contribution of sialic acid residues and the polylactosamine sequence(s) to the inhibition of the ligand binding property of MPR-2A appears independent, i.e. when MPR-2A was treated with Endo-β-Galase and neuraminidase, the increase in binding to immobilized ligand was nearly additive (Fig. 10). The mechanism by which polylactosamine and sialic acid residues affect the binding affinity of MPR-2A is not immediately apparent. One possibility is that sialic acid residues and the polylactosamine sequence(s) are located in the vicinity of the Man-6-P binding pocket and exert steric interference and/or an electrostatic effect. Alternatively, the presence of these components may alter the conformation of the receptor such that its affinity for Man-6-P-containing ligands is lowered.

Hoflack and Kornfeld (4) previously reported that the enzymatic deglycosylation of MPR-2 abolished its affinity for Man-6-P-containing ligands. Hille et al. (9) found that MPR-2 synthesized in the presence of tunicamycin or translated in vitro in the presence of competitive glycosylation inhibitors lacked the ability to bind ligands. These authors proposed that oligosaccharide chains may either stabilize the conformation of the receptor or participate directly in the binding of ligand. In contrast to these findings, Wendland et al. (10) found that N-glycosylation is not required for ligand binding activity, but instead may play an important role in maintaining the stability of the receptor. In light of these results and those described in this manuscript, it is suggestive that the N-linked oligosaccharides associated with each glycosylation isoform exert differing effects on the ligand binding activity of MPR-2, depending on the degree and type of glycosylation. Core glycosylation of the receptor may be required for the acquisition of ligand binding activity and/or for the maintenance of active conformation, while further processing of the associated oligosaccharide chains (i.e. acquisition of polylac-
Glycosylation Isoforms of Low Molecular Weight Man-6-P Receptor

tosamine sequences and sialic acid residues) lowers the affinity of the receptor for ligand. It has been proposed that MPR-2 participates both in the delivery of lysosomal enzymes to lysosomes and in the secretion of these enzymes to the extracellular milieu, depending on the location where the enzyme-receptor complex dissociates (3, 47). It is tempting to speculate that altering the receptor-ligand interaction by modifying carbohydrate components (i.e. polylactosamine sequences and sialic acid residues) may in turn alter the dissociation sites, which in turn may affect the targeting/secretion functions of the receptor.

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