CHAPTER II

BIOCHEMICAL CHARACTERIZATION OF NON-MAMMALIAN VERTEBRATE/INVERTEBRATE MPRs.
2.0 INTRODUCTION

It is well established that two transmembrane glycoproteins designated as MPR 300 and MPR 46 are mannose 6-phosphate specific receptors that mediate the transport of lysosomal enzymes to lysosomes in eukaryotic cells. The products of acid hydrolysis of O-phosphonomannan, phosphomannan core (PM) and pentamannosyl phosphate (PMP) have been used as functional appendages in affinity chromatography to purify the receptors. Both the receptors have been purified from the membrane extracts of bovine and human liver tissues on PM coupled to cyanogen bromide activated Sepharose gel in presence of divalent cations. The receptors have been separated from each other by passing the mixture onto another affinity matrix Dictyostelium discoideum lysosomal enzyme secretions coupled to Affigel-15 (E-Affigel) in presence of EDTA (Hoflack and Kornfeld., 1985; Stein et al., 1987a). The objective of the present study was to identify these proteins from non-mammalian vertebrates and invertebrates and to develop alternative affinity matrices for the purification of MPRs and gain further insight into the evolution of these receptors.

Here we report on the development of new affinity matrices Sepharose-divinyl sulfone-PM (PM gel) (Udaya lakshmi and Siva kumar., 1996) and Sepharose-divinyl sulfone-PMP (PMP gel) (Siva Kumar., 1996) and their use to purify both MPR proteins or only the MPR 300 from goat employing appropriate buffer conditions. In the present study, a new affinity method has been used for the efficient separation of the two receptors (goat MPR 300 and MPR 46) which is comparable to the earlier conventional method. Additionally the PM gel has been used to purify the MPR 300 from fish and the invertebrate Unio. Although MPR 46 from chicken and fish was detectable by direct immunoprecipitation with MPR 46 specific α-MSc1 antibodies, it failed to bind on PM gel under standard buffer conditions used to purify mammalian receptors. Therefore various modified buffer conditions were used to optimize the binding of MPR 46 from metabolically labeled chicken and fish cells to PM gel. The present biochemical study has helped us greatly to expand the knowledge about the occurrence of MPRs in animal kingdom and their binding abilities to phosphomannan gel.
2.1 MATERIALS

2.1a Instruments:

HPLC
SMART-System
Gels for SMART
Gel filtration column-fast desalting PC 3.2/10 (3.2 x 100mm)
Reverse phase HPLC Column Aquapore RP-300 (C8)
(2.1 x 220 mm)
UV detector for SMART
μ Peak detector
PTH aminoacid analyzer model 120A
Film developing machine model Gevamatic 60
Heating block thermostat 5320
Liquid scintillation counter model 1900 TR
Confocal laser scanning microscope
Phosphorimager, IPR 1000
Pipetman 20,200,1000µl

Spectrophotometer, UV 160A
Vacuum concentrator speed vac. SVC 100H

2.1b Chemicals:

Acetone
Acetonitrile, HPLC
30% Acrylamide/ 0.8% Bisacrylamide
(NN' methylenebisacrylamide)
Acetic acid
Ammonium acetate
BCA
BSA
Bromophenol blue
Calcium chloride (CaCl2)
Chloroform
Dimethysulfoxide ultra pure (DMSO)
PPO

Pharmacia, Uppsala, Sweden
Pharmacia, Uppsala, Sweden
Applied Biosystems (AB1)
Foster City, USA
Applied Biosystems, Foster City, USA
Agfa-Gevaert, Leverkusen
Eppendorf, Hamburg
Packard, Frankfurt
Zeiss, Oberkochen
Fuji, Tokyo, Japan
Gilson Medical Electronics, Villiersle-Bel, France
Schimadzu, Kyoto, Japan
Savant instruments, Farmingdale
Merck
Baker
Roth
Merck
Fluka
Sigma
Biomol, Hamburg
BioRad
Merck
Merck
Roth
DTT
EDTA
Ethanol
Ethanolamine
Formaldehyde (37%)
Freund's complete/ incomplete adjuvant
Glucose 6-phosphate
Glycerin
Glycine
Guanidium hydrochloride
HPLC grade water
HEPES
Imidazole
Iodoacetic acid (IAA)
Iodogen
Liquid Scintillation fluid
p-M.E
Methanol
Mowiol
Nonidet-40 (NP 40)
Pansorbin cell suspension
(Heat inactivated *staphylococcus aureus* cell suspension)
Paraformaldehyde
Phenylmethylsulfonylfluoride (PMSF)
Prestained high molecular weight protein markers
Propylgallatt (3,4,5-Trihydroxybenzoic acid propylester)
Saponin
Sepharose 6B
SDS
TEMED
TCA
TFA
Tris-(hydroxymethyl)-aminoethane (Tris)
Triton X-100
Tween 20
Serva
Merck
Merck
Sigma
Merck
Sigma
Sigma
Merck
Roth
Fluka
Baker
Serva
Merck
Serva, Heidelberg
Pierce Co.
Roth
Sigma
Merck
Calbiochem
Sigma, Deisenhofen
Calbiochem
Sigma
Serva, Heidelberg
Calbiochem
Serva
Sigma, Deisenhofen
Pharmacia
Sigma
Sigma
Merck
Fluka
Roth
Sigma, Deisenhofen
Sigma, Deisenhofen
2.2 METHODS

2.2.1 Hydrolysis of $O$-Phosphonomannan Y-2448:

This was carried out according to Bretthauer et al., (1973). 2.5 gm of $O$-phosphonomannan (from yeast hansenula holstii) was suspended in 50 ml of water in a screw cap bottle and left overnight for swelling. 500 mg of KCl was added and the pH of the suspension adjusted to 2.4 with acetic acid. The contents were then hydrolyzed in a boiling water bath at $100^\circ$C for 60 min. The suspension was cooled to room temperature and centrifuged at 10,000 rpm for 30 min to remove any insoluble material. The clear supernatant was neutralized to pH 11.0 with saturated barium hydroxide. To this an equal volume of 95% ethanol was added and left overnight at 4°C. The precipitated phosphomannan core was collected by centrifugation at 10,000 rpm for 30 min. The pelleted phosphomannan core (PM) dissolved in water and made acidic with acetic acid, dialyzed against water and lyophilized. To the supernatant which contains the pentamannosyl phosphate (PMP) was added an equal volume of ethanol and allowed to stand for one to two hours on ice. The suspension was centrifuged at 10,000 rpm for 30 min and the pellet re-dissolved in water with mild acidification and desalted by addition of Dowex 50 resin. The resin was removed and the solution lyophilized.

2.2.2 Preparation of Affinity Matrices:

Activation of Sepharose with Divinyl sulfone:

20 ml Sepharose 6B was washed thoroughly with distilled water on a sintered glass funnel and the wet cake was suspended in 20 ml of 0.5 M sodium carbonate/ bicarbonate buffer pH 11.0. 2.0 ml of divinyl sulfone was added and the suspension was shaken gently at room temperature for 70 min and washed thoroughly with distilled water on a sintered glass funnel. The activated gel was separated into two 10 ml portions.
Coupling of Phosphomannan Core and Pentamannosyl phosphate to the Activated gel:

Phosphomannan core and pentamannosyl phosphate obtained from the hydrolysis of O-phosphonomannan were coupled separately as functional appendages to the activated gel. The activated gel (10 ml) was washed with 0.5 M sodium carbonate/bicarbonate buffer pH 10.0 on a sintered glass funnel and the wet cake was suspended in 10 ml of carbonate buffer pH 10.0 containing 200 mg of phosphomannan core. The remaining 10 ml portion of the activated gel was processed as above and the wet cake was suspended in carbonate buffer pH 10.0 containing 200 mg of PMP. Coupling was allowed to proceed in cold for 24 hours. At the end of the coupling reaction the gels were separately passed through a sintered glass funnel and the solution obtained was saved to determine the extent of binding. The gels were washed with deionised water and finally suspended in 0.5 M sodium bicarbonate buffer pH 8.5 containing 0.2 ml of β-mercaptoethanol and mixed at room temperature for 3 hours. The gels were finally washed with distilled water and stored at 4°C in column buffer until further use. The extent of PM or PMP coupled to Sepharose was determined as mannose equivalents as described (Dubois et al., 1956).

2.2.3 Preparation of Acetone Powder:

Reagents used: (All steps were performed at 4°C).

- 0.5 M CaCl₂ and 1 mM NaHCO₃
- 4 N acetic acid
- Chilled acetone
- Diethyl ether

Acetone powder was prepared following the protocol of Distler and Jourdian (1987). Liver tissue (goat and fish) or whole animal tissue (Unio) was diced, homogenized for 1 min in waring blender with 1.6 vol. of 0.5 mM CaCl₂ and 1 mM NaHCO₃. pH was adjusted to 5.0 by drop
wise addition of 4 N acetic acid. The suspension was centrifuged for 15 min at 9000 rpm and the pellet was resuspended in 2.4 vol. of the same buffer and pH adjusted to 5.0, centrifuged at 9000 rpm for 15 min. Pellet was homogenized for 1 min in waring blender with 6 vol. of chilled (-20°C) acetone. The suspension was filtered rapidly through Whatmann 3 MM filter paper placed over a Buchner funnel. Reddish brown cake obtained was re-extracted with occasional pulverization to remove ether. Dry powder obtained was stored at -80°C until use.

2.2.4 Extraction of Membrane Proteins and Purification of MPRs:

Note: All operations mentioned below were performed at 4°C.

Buffer used:

- **Buffer 1**: 50 mM imidazole-HCl pH 5.0, 150 mM NaCl, 0.5 mM CaCl₂ and 0.1 mM PMSF
- **Buffer 2**: 50 mM Sodium acetate pH 4.6, 150 mM NaCl, 0.5 mM CaCl₂
- **Buffer 3**: 50 mM imidazole-HCl pH 7.0, 5 mM Sodium β-glycerophosphate and 150 mM NaCl
- **Buffer 4**: Buffer 3 containing 0.05% Triton X-100 and 10 mM MnCl₂
- **Buffer 5**: Buffer 3 containing 0.05% Triton X-100 and 2 mM EDTA

Fresh liver tissue freed from the connective tissue or the acetone powder prepared as above was homogenized with 6 vol. of buffer 1 in a waring blender and stirred overnight. The suspension was centrifuged at 9000 rpm for 15 min and the supernatant discarded. The pellet obtained was resuspended in 6 vol. of buffer 2 and homogenized well in a waring blender and centrifuged. The pellet was suspended in 6 vol. (to the pellet weight) of buffer 3, homogenized in a waring blender and the suspension was kept stirring in cold to which Deoxycholate and Triton X-100
were added to final concentration of 0.1% and 1% respectively. The suspension was allowed to stir overnight. The suspension was then centrifuged at 4000 rpm for 15 min. and the clear supernatant containing the membrane proteins was collected. To this was added MnCl₂ (final concentration 10 mM) and the suspension was stirred for 60 min. and centrifuged at 9000 rpm for 45 min. The clear supernatant obtained served as the ready source of the mannose 6-phosphate receptors.

2.2.5 Purification of MPRs by Affinity Chromatography:
The membrane proteins extract from fresh liver tissue or the acetone powder was loaded on affinity matrices Sepharose-DVS-PM pre-equilibrated with buffer 4 or Sepharose-DVS-PMP pre-equilibrated with buffer 5. The columns were run at a flow rate of 30 ml/hr, and subsequently washed extensively with the respective buffers. Elution was first performed with 5 mM glucose-6-phosphate to ensure any non specific elution of the proteins, followed by 5 mM mannose 6-phosphate in buffer 4 or 5. The mannose 6-phosphate eluates were pooled and dialyzed extensively against buffer 4 or 5 and concentrated using Amicon concentrator.

2.2.6 Protein Estimation:
Protein estimation was done using BCA reagent employing bovine serum albumin as a standard following manufacturer's instructions.

BCA Reagent: 10 ml of BCA and 0.5 ml of 4% CuSO₄.

The volume of the TCA precipitated protein (neutralized with 20 μl of 1 M Tris) sample needed for estimation was made up to 500 μl with distilled water and mixed with 1 ml of BCA reagent, incubated at 37°C for 30 min. O.D was measured at 562 nm.
2.2.7 SDS-Poly Acrylamide Gel Electrophoresis:

The electrophoretic separation of proteins was done by discontinuous SDS-PAGE according to Laemmli (1970).

Anode buffer: 50 mM Tris-HCl pH 8.6, 384 mM Glycine in double distilled water

Cathode buffer: 0.1% (w/v) SDS, 0.001% (w/v) Bromophenol blue in anode buffer

2x Sample buffer (reducing): 2% (w/v) SDS, 20% (v/v) Glycerin, 250 mM Tris-HCl buffer pH 6.8 (20 mM Dithiothreitol).

Resolving gel buffer: 0.4% SDS, 1.5 M Tris-HCl pH 8.8

Stacking gel buffer: 0.4% (w/v) SDS, 0.5M Tris-HCl pH 6.8

Acrylamide: 30% (w/v) Acrylamide, 0.8% (w/v) N N’ methylenebisacrylamide

Ammoniumperoxide sulfate: 10% (w/v) in water

The amounts of reagents to be used for different percentage of resolving gel and stacking gel are shown in the Table 4 and Table 5 respectively. Resolving gel reagents were mixed (APS and TEMED must be added at the end) and poured into the sealed glass plates (size: 16 cm x 18 cm, 1mm Spacer). Overlaid with water saturated butanol, allowed to polymerize for 30 min at room temperature, butanol was removed and the gel was rinsed with water. Stacking gel solution was poured, a comb with required number of wells inserted and allowed to polymerize for 30 min. Wells were rinsed with water. The samples were cooked at 95°C for 5 minutes with sample buffer mixed in 1:1 ratio, centrifuged at 14,000 rpm for 2 min, supernatant loaded into the wells. These were overlaid with cathode buffer and placed in the electrophoretic chamber. Samples were subjected to electrophoresis for 2-3 h at 50 mA at 4°C.
Table 4: Reagents used for the preparation of resolving gel with different polyacrylamide concentration

<table>
<thead>
<tr>
<th>% of PAA gel</th>
<th>6%</th>
<th>7.5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (ml)</td>
<td>6.3</td>
<td>7.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Resolving gel buffer (ml)</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APS (μl)</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>adj. 30 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Reagents used for the stacking gel

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (ml)</td>
<td>1.3</td>
</tr>
<tr>
<td>Stacking gel buffer (ml)</td>
<td>2.5</td>
</tr>
<tr>
<td>APS (μl)</td>
<td>100</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>10</td>
</tr>
<tr>
<td>H₂O (ml)</td>
<td>6.1</td>
</tr>
</tbody>
</table>

2.2.8 Silver Staining: Ansorge (1985)

Methanol, CuCl₂·H₂O, TCA, KMnO₄, AgNO₃, K₂CO₃, Formaldehyde 37%.

Solutions for the big gel:

1. Fixative: 75 ml methanol, 18 g TCA, 3 g CuCl₂ adjusted to 150 ml with water (stored at 4°C in brown bottle and can be reused for 4-5 times)

2. Solution A: 50 ml ethanol (10%), 25 ml 100% acetic acid (5%) adjusted to 500 ml with water.

3. 15 mg KMnO₄ in 150 ml water (freshly prepared)
4. 150 mg AgNO₃ in 150 ml water (freshly prepared, store in brown bottle)

5. 10% ethanol

6. 10 g K₂CO₃ in 200 ml water (freshly prepared)

7. 60 ml 5% K₂CO₃ from 6, 60 ul formaldehyde water to 300 ml (prepared just before use)

Staining protocol at room temperature with constant shaking:

Fixation from 60 min to overnight. After fixation, the following steps are indicated in the Table 6.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Big gel</th>
<th>Mini gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>15 min</td>
<td>15 min</td>
</tr>
<tr>
<td>KMnO₄</td>
<td>15 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Water (300 ml)</td>
<td>1-2 min</td>
<td>1-2 min</td>
</tr>
<tr>
<td>Solution A</td>
<td>15 min</td>
<td>15 min</td>
</tr>
<tr>
<td>10% ethanol</td>
<td>15 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Water</td>
<td>15 min</td>
<td>10 min</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>30 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Water</td>
<td>1-2 min</td>
<td>1-2 min</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>2 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Developing with solution 7</td>
<td>5-10 min</td>
<td>5-10 min</td>
</tr>
</tbody>
</table>

Table 6: Sequential Incubation steps with various reagents followed for silver staining of the proteins resolved on polyacrylamide gels

Reaction stopped with 10 min incubation in solution A, gel was then washed twice with water each with 30 min incubation, and gel was dried in the gel dryer.
2.2.9 Raising Antibodies to the Purified Receptor:

Antibodies to the affinity-purified protein (goat liver MPR 300 and *unio* MPR 300) were raised in a rabbit. 250 µg of the protein in 0.5 ml was emulsified with 500 µl of Freund's complete adjuvant and injected subcutaneously into a rabbit. Rabbit received a booster dose in the 3rd and 5th week (incomplete adjuvant). Rabbit was bled 10 days after the 3rd injection and the blood was allowed to clot, serum was separated by centrifugation and stored at -20°C in aliquots.

2.2.10 Iodination of Proteins:

*Fraker* and Speck (1978)

Reagents:

1. 20 mM Borate buffer pH 8.0 containing 0.05% Triton X-100 and 5 mM mannose 6-phosphate.

2. Iodo-gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) solution: 0.8 mg of Iodogen (M.W 432.06) was directly weighed into an eppendorf tube, dissolved in 577 ul of dichloromethane. 200 ul of it was transferred to another tube and diluted 1-3 times (400 ul) to get final 1mM concentration. (Iodogen rapidly initiates iodination, the thin film of iodogen on the reaction tubes minimizes the direct exposure of the protein to the oxidant).

3. Iodogen tubes: 50-80 ul of iodogen solution was uniformly coated under Nitrogen atmosphere at the bottom of a conical glass centrifuge tubes (for each iodination, 2 tubes are sufficient).

4. Column buffer: 1 mg/ml Potassium iodide, 0.05% BSA, 0.05% Triton X-100 in 10 mM PBS. Mannose 6-phosphate eluate was concentrated in an *Amicon* concentrator (10 kDa cut off membrane). The concentrated protein was then acetone precipitated with chilled (-20°C) acetone (to final 80% concentration), incubated overnight at -20°C. The sample in eppendorf tubes was centrifuged at 14,000 rpm for 10 min, acetone removed and the protein pellet was air dried by leaving the tubes open on ice. The pellet (1-10 µg) was suspended in 60 ul of borate
buffer and sonicated (on ice) to disturb any aggregates. The sample was centrifuged briefly and clear supernatant transferred to a fresh conical glass tube numbered as tube 1. To this 1 ul (100 μCi) of $^{125}\text{I}$ was added under the hood, tube was closed with parafilm and incubated on ice for 5 min (a timer has to be set from the start of addition of Na$^{125}\text{I}$). The contents were transferred to tube 2 (iodogen tube) and incubated with rotation on ice for 8 min. The reaction was stopped by transferring the contents to tube 3 (conical glass tube) containing 10 ul of 20 mM β-M.E. Tube 2 was rinsed with 130 ul of column buffer and transferred to tube 3. Additional 300 ul of column buffer was added to tube 3 to make up the volume to 500 ul

The iodinated protein fraction was separated from the free iodine by passing the sample over a 5 ml gel filtration SG-25 column pre-equilibrated with 20 ml of column buffer. The sample was loaded on the column and 10 fractions each of 0.5 ml were collected in eppendorf tubes placed in the lead aluminum metal holder. 2 ul from each of the tube was transferred to fresh eppendorf tubes numbered from 1-10 and counted for 1 min in a γ-ray counter.

2.2.11 Analysis of the Iodinated Protein Samples:

2.2.11a TCA Precipitation of Iodinated Protein:

From the peak fractions (usually 7and 8), about 100,000 cpm counts were taken in an eppendorf tube, volume made up to 100 ul with column buffer, 1 μg (10 mg/ ml BSA stock) of BSA and TCA (72%) to final 10% concentration were added, incubated on ice for 30 min. The sample was centrifuged at 14,000 rpm for 10 min, supernatant transferred to another tube. Both pellet and supernatant were counted in a γ-ray counter.

Pellet was then suspended in 20-30 μl of 1M Tris and equal volume of 2x sample buffer (+/- SH buffer) was added, cooked at 95°C for 5 min. The samples were centrifuged briefly and
subjected to SDS-PAGE analysis. The gel was put (glass container) in the fixative (50% methanol, 10% acetic acid in water) for 30-60 min, washed with water 3 times and finally placed in the saran wrap, air dried and exposed overnight to Roentgen film at -70°C.

2.2.11b Repurification of Iodinated Receptor Protein:

1 ml of PM gel packed in 5 ml sintered column was used. The gel was equilibrated with equilibration buffer (50 mM imidazole-HCl pH 7.0, 150 mM NaCl, 5 mM sodium 3-glycerophosphate, 10 mM MnCl₂ and 0.05% Triton X-100). A fixed amount of iodinated receptor protein corresponding to 100,000 to 200,000 cpm was taken in an eppendorf tube, volume was made up to 500 ul with column buffer and loaded on to the column. Unbound fraction was collected, followed by 10 wash fractions each of 500 ul. The fractions were counted in y-ray counter, when the counts were equal to background counts, elution was done first with 5 mM glucose 6-phosphate (5 fractions of 500 ul each) followed by 5 mM mannose 6-phosphate (5 fractions of 500 ul each). The G6P and M6P fractions were counted and the mannose 6-phosphate eulates were pooled, counted and dialyzed against column buffer to remove the mannose 6-phosphate. The re-purified receptor was used for the determination of pH optimum for binding to PM gel using analytical 0.2 ml PM gel and for immunoprecipitation.

2.2.11c Determination of pH Optimum:

**Buffer A**: 50 mM sod.acetate, 150 mM NaCl, 5 mM sodium p-Glycerophosphate, 0.05% Triton X-100, 10 µg/ ml BSA, 10 mM MnCl₂

**Buffer B**: 50 mM imidazole, 150 mM NaCl, 5 mM sodium P-Glycerophosphate, 0.05% Triton X-100, 10µg/ ml BSA, 10 mM MnCl₂ (Table 7)
Table 7: Column buffers with varied pH used for equilibration of PM gel

<table>
<thead>
<tr>
<th>Column number</th>
<th>PH</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>5.5</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>6.5</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>7.0</td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>7.5</td>
<td>B</td>
</tr>
</tbody>
</table>

Analytical PM gel (0.2 ml) packed in 1 ml sintered columns (total 8 columns) were pre-equilibrated with 10 ml of the respective pH buffers. A fixed number of counts (10,000-11,000 cpm) of iodinated receptor were used for each column, the sample was diluted to 200 ul with the respective column buffers. The unbound fraction (200 µl) was collected and the column was washed with 10 fractions each of 200 µl, and sequentially eluted with 5 mM glucose 6-phosphate and 5 mM mannose 6-phosphate (made in the respective column buffers) into 4 fractions each of 200 ul. The trichloroacetic acid precipitable radioactivity in the column fractions was monitored. In addition, 10,000 cpm were used for the direct TCA precipitation and the TCA precipitable counts were taken as 100%.

2.2.11d Immunoprecipitation of the Iodinated MPR Protein:

A fixed number of counts (80,000-100,000 cpm) of the iodinated receptor were taken into two eppendorf tubes labeled as PI (pre immune) and IM (immune). To this 2 ul of pre immune serum or immune serum (for MPR 300) or 10 ug of affinity-purified MSC1 antibody (for MPR 46) was added, volume was made up to 400 ul with PBS-Tween buffer (10mM PBS and 0.05%
Tween). The tubes were then incubated with rotation at 4°C for 2 hours. To pellet down the Ag-Ab immune complex, 40 μl of Pansorbin suspension was added, incubated with rotation for 1 hour at 4°C and centrifuged at 14,000 rpm for 2 min, both pellets and supernatants were counted. The Pansorbin pellets were then washed with 1 ml of PBS containing 0.05% Tween 4 times (pellet was disturbed by agitation before addition of the buffer for thorough washing). After the final wash, Pansorbin pellets were cooked in 60 μl of sample buffer at 95°C for 5 min, centrifuged and the clear supernatants were subjected to SDS-PAGE analysis.

2.2.12. Metabolic Labeling of Cells and Extraction of Membrane Proteins:

Buffers:

1. 0.1 M sodium acetate buffer pH 6.0 (adjusted with acetic acid) containing 0.2 M NaCl. PMSF, Iodoacetic acid and EDTA were added to the buffer just before use to a final concentration of 1 mM, 5 mM, and 1 mM respectively.

2. 50 mM imidazole-HCl pH 6.0/6.5/7.0 and 150 mM NaCl for column equilibration.

3. Imidazole buffer containing 0.05% Triton X-100 (wash buffer).

4. Elution buffers: 5 mM glucose 6 phosphate (30.4 mg/ml = 100 mM stock in equilibration buffer) 5 mM mannose 6-phosphate (7.91 mg/ml = 20 mM stock in equilibration buffer)

*Xiphophorus* fish embryonic cells (A2) were cultured in Hams F12 medium (GIBCO BRL) containing 10% fetal calf serum at 28°C in 95% air / 5% CO₂ (Kuhn *et al.*, 1979). Cells were grown in mono layers on 6 cm plates, incubated in methionine and cysteine free medium (hunger medium) for 1 hour and 400 μCi (40 μl) 35S methionine and cysteine mixture was added. Labeling was done for 8 hours. Chicken embryonic fibroblast cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal calf serum. The cells were grown at 37°C in 5% CO₂ incubator, they were metabolically labeled for 12-14 hours with 35S methionine and cysteine.
After labeling, cells were scraped from the plates using a rubber policeman, and the labeled cell pellets were collected and homogenized by sonication in 1.0 ml of 0.1 M sodium acetate buffer pH 6.0 to extract soluble proteins. After sonication, the cell lysate was centrifuged at 109,000 x g for 30 min (50,000 rpm, TLA 100.3 rotor). The membrane pellet obtained was then processed for extraction of the membrane proteins in 0.8 ml of 50 mM imidazole-HCl buffer of varied pH range (6.0-7.0) containing 0.5% Triton X-100, 1mM EDTA, 5mM iodoacetic acid, and 1mM PMSF. To the labeled membrane extract, divalent cations were added to a final concentration of 10 mM (details as given in Table 8), incubated on ice for 15 min, and centrifuged at 109,000g for 20 min. The clear membrane protein extract (0.8 ml) was subjected to direct immunoprecipitation for the detection of MPRs or subjected to PM gel analytical (0.2 ml gel packed in 1 ml column) affinity chromatography. The column was washed with wash buffer (6-8 x 0.8 ml fractions) and eluted with 5mM glucose 6-phosphate (5 x 0.8 ml fractions) and 5 mM mannose 6-phosphate (5 x 0.8 ml fractions). The column fractions (unbound, first wash, G6P 1 and 2 and M6P land 2) were then used for immunoprecipitation of MPRs with MPR specific antibodies.

**Antibodies used for the detection of MPRs:**

**MPR 46** Rabbit α-MSC1 antibodies raised against highly conserved cytoplasmic tail of human MPR 46 (Klumperman et al., 1993).

**MPR 300** Rabbit a-goat MPR 300 antibodies (Udaya lakshmi and Siva kumar., 1996) and rabbit anti- Unio MPR 300 antibodies.

**2.2.12a Immuno Precipitation of Metabolically Labeled Proteins:**

According to Kessler (1976)

Reagents used:

Protease inhibitors: 1 mM EDTA (stock 0.2 M in water), 1 mM PMSF (stock 0.2 M in methanol), and 5 mM IAA (stock 0.5 M in water)

Wash immunomix (IMM): 1% (w/v) Triton-X-100, 0.5% sodium-Deoxycholate in PBS
2 M-KCl-Immunomix: 2 M KCl in wash immunomix

Precipitation immunomix: 0.2% (w/v) SDS, 10% (w/v) BSA, 1 mM EDTA, 1 mM PMSF, 5 mM IAA in wash immunomix.

Neufeld buffer: 0.05% (w/v) NP-40, 0.1% (w/v) SDS, 0.6 M NaCl, 10 mM Tris-HCl pH 8.5.

Pansorbin: Required amount of Pansorbin (Calbiochem) suspension was taken and pelleted (14,000 rpm for 10 min), the pellet was suspended in the equal volume of wash immunomix.

Pre-adsorbed Pansorbin: Pansorbin was pre-adsorbed with cold membrane protein extract of respective cell lines and suspended in equal volumes of wash immunomix.

To one volume of the sample (0.8 ml) 0.8 ml of immunomix, 2 ul of pre immune serum and 100 ul of pansorbin suspension were added and was incubated with rotation for 2-4 hours at 4°C. It was then centrifuged at 14,000 rpm for 5 min, supernatant was then transferred to a new 1.5 ml eppendorf tubes (labeled properly) and 100 ul of Pansorbin was added, incubated with rotation at 4°C overnight, centrifuged at 109,000 × g /50,000 rpm (TLA 100.3 rotor) for 20 min. The supernatant was carefully (leaving 20-25 ul) transferred to fresh eppendorf tubes, MPRs were immunoprecipitated with 10 ug of affinity purified antibody (a MSC1 antibody for MPR 46) or 2 ul of antiserum and incubated with rotation at 4°C for 2 hours to overnight. To this was added 20 ul of preadsorbed pansorbin and after incubation for 1 hour at 4°C, the Ag-Ab complex was pelleted by centrifugation at 14,000 rpm in cold for 1 min. The supernatant was either discarded or used for another immunoprecipitation. The pansorbin pellets were washed at room temperature using ice cold buffers as given below.

The pellets were first disturbed by agitation, then suspended in 1 ml of Neufeld buffer (RT), mixed well to get uniform suspension, centrifuged at 14,000 rpm for 1 min, supernatant aspirated, pellet was disturbed and suspended in 1 ml of ice cold wash immunomix, uniform
suspension was made and centrifuged. The pellets were then sequentially washed as above with 1 ml of 2 M KCl in immunomix, followed by washing twice with 1 ml of 1 to 10 diluted PBS. The immune complexes were extracted from the pellets by cooking at 95°C for 5 min with 60 µl of sample buffer (-SH/+SH depending on the requirement) followed by centrifugation and the clear supernatant was subjected to SDS-PAGE. The gel was then fixed, dried and exposed to Roentgen film/phosphor imager screen or processed for fluorography for the detection of protein bands. Exposure time varied (24 hours-one week), depending on the signal intensity.

2.2.13 Fluorography:

According to Bonner and Lasky (1974)

For the amplification of radioactive signal (for β radiation), the radiolabeled proteins separated on polyacrylamide gels were impregnated with PPO (scintillator) and was then exposed to pre-flashed Roentgen film was as given below.

Fixative 50% methanol, 10%(v/v) acetic acid in water
DMSO bath 1-3 DMSO
PPO bath 20% (w/v) PPO in DMSO

The polyacrylamide gel was first incubated in the fixative for 30 min, then carefully passed (with the help of wooden forceps) sequentially through DMSO bath 1,2 and 3 for dehydration, each with an incubation time of 20 min. Then the gel was incubated in PPO bath for 3 hours to overnight. The PPO on the gel was precipitated with a water wash (the gel turns white). The gel was then placed in Saran Wrap and dried. The dried gel was then exposed to pre-flashed Roentgen film (Kodak XAR-5) and stored at -70°C or alternatively the gel was also exposed to phosphorimaging screen, and was scanned in the phosphorimager, and quantification of the bands was done using the software program, the quant analysis.
2.2.14 Deglycosylation:

Keinanen et al., 1988

PNGase F: Concentration of the enzyme: 100 m.units / 0.5 ul.

(The treatment of a glycoprotein with this enzyme leads to complete deglycosylation).

PNGase buffer: 0.1 M sodium phosphate buffer pH 8.6, 1.2% NP 40, 0.2% SDS, 1% p. M.E

The Pansorbin pellets of Ag-Ab immune complex (^{35}S-MPR 46 Ag and a MSC1 Ab) were cooked with 30 ul of PNGase buffer at 95°C for 5 min, centrifuged at 13,000 rpm for 10 min. The supernatant transferred to a fresh Eppendorf tube and to this 30 ul of PNGase buffer containing 2 mM PMSF, 10 mM IAA, 2 mM EDTA was added. The final 60 ul sample was divided into two equal portions and labeled as tube 1 and tube 2. One of them was used as a control and to the other 0.5 ul of PNGase F enzyme was added, incubated at 37°C for 16 hours and subjected to 10% SDS-PAGE.

2.2.15 Intracellular Immunofluorescence Staining:

Reagents:

1. 0.1 mg / ml Polylysine in H₂O, sterile filtered.
2. PBS.
3. Ca-Mg-PBS: 0.9 mM CaCl₂+ 0.5 mM MgCl₂ in PBS.
4. pFA: 3 g of paraformaldehyde weighed under the hood, suspended in 100 ml PBS and dissolved by heating at 80°C with constant stirring under the hood. pH was controlled with a pH paper to 7.4, filter sterilized, stored in small aliquots at -20°C.
5. 50 mM NH₄Cl in PBS.
6. 0.1 % Saponin in PBS (5% stock prepared and stored in aliquots at -20°C).
Mowiol (25 mg/ml stock): 20 g Mowiol 4-88 suspended in 80 ml PBS, stirred at RT for 24 h, to this 40 ml of 100% glycerin added, stirred for an additional 24 h at RT. pH was controlled with pH paper to 6-7. The above solution centrifuged at 12,000 rpm for 15 min at 20°C. Supernatant was stored in small aliquots at -20°C.

Antibleaching agent: 25 mg/ml Propylgallat was prepared 0.5 g of propylgallat dissolved in 10 ml glycerin, to this 10 ml of 10 mM Tris-HCl pH 8.0, 150 mM NaCl was added and pH was controlled at 6.0 using pH paper.

Mounting media: 1 vol propylgallat and 9 vol Mowiol mixed well (stock solutions were heated at 95°C for 5-10 min).

Fish Cells and BHK wild type 6 cells (over expressing human MPR 46) were grown to sub-confluency on poly-lysine coated glass coverslips placed in a 4 well plate. Fish cells were grown under growth conditions as described earlier and BHK WT6 cells were grown in DMEM containing 5% fetal calf serum and puromycin (5 µg/ml) at 37°C in 5% CO₂ incubator. Cells were washed with Ca-Mg-PBS, fixed with 200 µl of 3% paraformaldehyde for 30 min at RT, washed twice with PBS. All the following steps were done at RT, washing each time with 0.5 ml of the buffer mentioned. The paraformaldehyde was quenched with 10 min incubation in 200 µl of NH₄Cl, washed twice with PBS. Cells were permeabilized by incubating in 0.1% Saponin for 5-15 min.

To detect intracellular MPR 46, cells were first incubated for 1 hour at RT with affinity purified anti-MSC1 antibody (40 µg/ml in 0.1% Saponin, centrifuged for 2 min), 20 µl of primary antibody was pipetted out onto a parafilm and coverslips were placed with the cells facing below. The coverslips were again put back into the 4 well plate with the cells above. Cells were washed 3 times with 0.1% Saponin each time with 5 min incubation. Cells were then incubated
for 1 hour with the secondary antibody, goat anti-rabbit IgG conjugated to Texas-Red (excitation wavelength 543 nm; diluted 1:200 in 0.1% Saponin) and were processed in a similar way as was done for the 1ºAb. After this incubation, cells were washed twice with PBS and once with sterile double distilled water. Finally the coverslips with the cells were embedded in 30 µl of mounting media placed over the glass slide and left overnight in dark at RT. Stored at 4°C until viewed under confocal laser scanning microscope, the final pictures were prepared in Adobe Photoshop.

2.2.16 Protein Sequencing:

Schmidt et al., 995 a

The purified Unio MPR 300 protein was acetone precipitated and processed for amino acid sequencing following the steps given below.

Various steps followed for the protein sequencing:

a. Reductive carboxymethylation (up to 100 µg of protein could be used)

b. Desalting on G-25 gel filtration column

c. Tryptic digestion of the carboxymethylated protein and separation of tryptic peptides by RP-HPLC.

d. Mass spectrometry of the peptides.

e. Peptide sequencing by automated Edman’s degradation method

2.2.16a Reductive Carboxymethylation of Unio MPR 300:

Carboxymethylation buffer: 6 M Guanidium hydrochloride

(CM buffer) 10 mM EDTA

400 mM Tris-HCl pH 8.6

DTT: 1 M

Iodoacetic acid: 2 M, pH 8.6 adjusted with ammonium hydroxide.
The acetone-precipitated affinity purified *Unio* MPR 300 (70 μg) was dissolved in 76 μl of CM buffer, the pellet was dissolved by sonication (optional). 4 μl of 0.2 M DTT/ H₂O (final 10 mM concentration) was added and was put under Argon gas for a while and then incubated for 60 min at 50-55°C. The sample was cooled to RT (very important step) and was treated in a sequential manner as shown below,

1. 6 μl 0.4 M Iodoacetic acid, under Argon, incubation at RT in the dark (IAA is light sensitive)
2. 6 μl of 0.2 M DTT / H₂O, under Argon, incubation at 50-55°C for 60 min, cooled to RT.
3. 6 μl of 0.4 M IAA, under Argon, incubation at RT for 30 min in the dark.
4. 6 μl of 0.2 M DTT / H₂O added and stored. This step was done only when the protein has to be stored.

### 2.2.16b Desalting on Sephadex-G-25 Column by HPLC:

Column buffer: 25 mM Ammonium acetate pH 8.6, 10% (v/v) Acetonitrile (to reduce the hydrophobic interaction of protein with matrix).

The sample was centrifuged for 10 min at 14,000 rpm and clear supernatant was used for injecting. The fractions were collected at a flow rate of 100 μl / min with the help of an automated fraction collector and the absorbency was measured at three different wavelengths of 280, 295 and 340 nm. The peak fraction was collected and the volume of the peak fraction was measured and was made upto 450 μl with column buffer.

### 2.2.16c Tryptic Digestion:

Tryptic digestion was carried out with the addition of TPCK-treated (Chymotrypsin inhibitor) trypsin (1 μg/μl) at a concentration of 2% (w/w) to the 70 μg of protein, incubated at 37°C for 16 hours.
2.2.16d Separation of *Unio* MPR 300 Tryptic Peptides by Reverse Phase-HPLC:

Buffer A: 0.1% (v/v) Trifluoroacetic acid in H₂O

Buffer B: 0.1% (v/v) Trifluoroacetic acid in 90% Acetonitrile.

After the tryptic digestion, the separation of the peptides was done by passing the sample over the Silica gel overlaid with the C8 alkylgroups using the RP-HPLC automated system. The column was eluted with the increasing concentration of acetonitrile (1% acetonitrile / min) at a flow rate of 300 μl / min, fractionation was performed by automatic peak recognition (OD 214 nm). The absorbency was measured at three different wavelengths, 214 nm (for peptide bonds), 280 nm (for Tryptophan and Tyrosine) and 295 nm (for Tryptophan). The purity and molecular mass of the peptides was determined by mass spectrometry.

2.2.16e N-Terminal Sequencing by Edman's Degradation Method:

Well separated, pure fragments were used for sequencing by Edman's degradation method using automated amino-acid sequence analyzer model All A. Sequencing was carried out according to Edmann and Begg. (1967).
2.3 RESULTS

2.3.1 Affinity Purification and Separation of Goat MPR proteins:

The membrane extract from the goat liver tissue was subjected to affinity chromatography on PM gel in the presence of divalent cations (Mn\(^{2+}/\)Mg\(^{2+}\)). After extensive washing, the column was eluted with 5 mM mannose 6-phosphate. In the 10% SDS-PAGE analysis of the mannose 6-phosphate eluate, both MPR 300 and MPR 46 were detected (Fig 6). About 1 mg of the receptors was purified to homogeneity by affinity chromatography of membrane extracts from 1 kg of goat liver tissue or 100 g of the goat liver acetone powder. For the separation of the two receptors, the mixture of MPRs was dialyzed extensively against buffer containing 2 mM EDTA and passed through PMP gel equilibrated with column buffer containing 2mM EDTA. The unbound fraction was collected and the column was eluted with 5mM mannose 6-phosphate. When aliquots of the column fractions were analyzed on SDS-PAGE, only MPR 46 was detected in the unbound fraction and only MPR 300 in the mannose 6-phosphate eluate (Fig 6). The separation of the two receptors was done in parallel with the conventional method of using E-Affiigel and the separation efficiency was found to be comparable (Fig 6).

2.3.2 Purification of Fish (trout) MPR Protein and pH Optimum:

Membrane proteins from trout liver were extracted following the protocol described under methods, the membrane extract was adjusted to 10 mM manganese chloride and clarified by centrifugation. The clear supernatant served as the source of receptors. Affinity chromatography of the membrane extract on PM gel was carried out as described under methods. The mannose 6-phosphate eluates from PM-Sepharose affinity chromatography were analyzed on SDS-PAGE followed by silver staining. A band with an apparent molecular mass of 300 kDa (the typical electrophoretic mobility of mammalian MPR 300) was detected and MPR 46 was not detected. From 200 g of fresh liver tissue about 10-15 μg of protein was
obtained. The available data do not allow to decide whether the concentration of MPR 46 in fish liver is too low to be detected or whether fish MPR 46 failed to bind to the affinity matrix under the conditions used.

To characterize the purified MPR 300 from fish liver in comparison to the receptor from goat liver, both receptors were radioiodinated. The apparent molecular mass of the non-reduced radioiodinated MPR 300 from both species was nearly identical (Fig 7). Under reducing conditions, the electrophoretic mobility of MPR 300 from both species decreased to the same extent (Fig 7). When radioiodinated fish liver MPR 300 protein was reapplied to PM gel as described under methods, MPR 300 was bound and could be eluted with 5 mM mannose 6-phosphate but not with glucose 6-phosphate (Fig 8). These results confirm that the MPR 300 protein from the liver tissue shows similar binding to phosphomannan as the mammalian MPR 300 protein. The optimal binding of fish MPR 300 to phosphomannan was found at pH 7.0 with lower binding in the range of pH 6.0-7.5 (Fig 9), as described earlier for mammalian MPR 300 (Hoflack et al., 1987), essentially no binding was detected at pH 5.5 or below.

2.3.3 Binding of Chicken MPR 46 to PM-Sepharose:

The presence of MPR proteins in chicken is very well established and they have been cDNA cloned and sequenced (Matzner et al., 1996). It was shown that MPR 300 from chicken binds to PM under physiological salt concentration as mammalian MPR proteins, whereas MPR 46 failed to bind to PM-Sepharose under similar conditions (Fig 10). Keeping in view of the three major factors (pH, ionic strength, presence of metal ions in case of MPR 46) which influence the binding of MPR to its ligands, a series of experiments were performed at various buffer conditions as listed in Table 8. Optimal binding was seen at pH 6.5 and at very low ionic strength (Table 9). The immunoprecipitates of the column fractions (unbound, first wash,
Figure 6: Separation of Large (MPR 300) and Small (MPR 46) Mannose 6-Phosphate Receptors. 10% SDS-PAGE analysis of the affinity purified and separated goat liver MPR proteins detected by silver staining. Lane 1, Mixture of MPRs purified on PM gel; Lane 2 and 3, unbound (MPR 46) and eluted (MPR 300) fractions from PMP-gel; Lane 4 and 5, unbound (MPR 46) and eluted (MPR 300) from E-Affigel/Dictyostelium discoideum lysosomal enzyme secretion coupled to Affigel-15.

Figure 7: Apparent Molecular Mass of Radioiodinated MPR 300 Under Reducing and Non-Reducing Conditions in SDS-PAGE: Apparent molecular mass of radioiodinated MPR 300 from trout liver (lanes 1 and 2) and goat liver (lanes 3 and 4) was analyzed on 6% acrylamide gel under non-reducing (Lanes 1 and 3) and reducing conditions (Lanes 2 and 4). Arrows indicate position of MPR 300 protein.
Figure 8: Re-chromatography of Radioiodinated Fish Liver MPR 300: The affinity purified fish liver MPR 300 was $^{125}$I-labeled and subjected to re-chromatography on PM gel. All the column fractions were analyzed by 6% SDS-PAGE. Lane 1, flow through; lane 2, 5 mM glucose 6-phosphate eluate; lane 3, 5 mM mannose 6-phosphate eluate. Arrow shows position of MPR 300.

Figure 9: Effect of pH on the Binding of Fish Liver MPR 300 to PM gel. Radioiodinated MPR 300 was bound to 0.2 ml of PM gel at varied pH range as described under methods. After extensive washing, columns were eluted with 5 mM mannose 6-phosphate (prepared in the respective pH buffers). The column fractions (both flow through and eluted) were subjected to TCA precipitation and both pellets and supernatant were quantitated in a $\gamma$-ray counter. Bound MPR 300 was calculated as percentage of TCA precipitable radioactivity in the 5 mM mannose 6-phosphate eluate relative to total TCA precipitable radioactivity recovered in all column fractions.
Figure 10: Binding of Chicken MPR 46 to PM Sepharose under Standard Buffer Condition. A membrane protein extract of $^{35}$S-metabolically labeled chicken embryonic fibroblasts was subjected to PM-Sepharose affinity chromatography under standard binding buffer condition (50 mM imidazole-HCl pH 7.0, 150 mM NaCl, 5 mM sodium p-glycerophosphate and 10 mM MgCl$_2$) and MPR 46 from the column fractions was immunoprecipitated with anti-MSCl antibody. Immunoprecipitates were analyzed by 10% SDS-PAGE under reducing conditions and detected by phosphorimaging. Lane 1, flow through; lane 2, first wash; lane 3 and 4, 5 mM glucose 6-phosphate eluate; lane 5 and 6, 5 mM mannose 6-phosphate eluate; lane 7, Direct immunoprecipitation of the equal amount used for chromatography.

Figure 11: Binding of Chicken MPR 46 to PM Sepharose at Optimized Buffer Condition. Membrane protein extract of $^{35}$S-metabolically labeled chicken cells was subjected to analytical affinity chromatography at pH 6.5 (minus salt, described under results). MPR 46 from the column fraction was precipitated with anti-MSCl antibody. Immunoprecipitates subjected to 10% SDS-PAGE followed by phosphorimaging. The amount of radioactivity in various fractions (unbound, glucose 6-p and mannose 6-p eluate) was quantitated by quant analysis in the phosphorimaging system. M-monomer and D-dimer.
Figure 10

Chicken MPR 46

Figure 11
glucose 6-phosphate and mannose 6-phosphate) obtained with MPR 46 tail specific anti-MSc1 antibody were analyzed on SDS-PAGE and detected by fluorography (Fig 11).

<table>
<thead>
<tr>
<th>Buffer composition</th>
<th>Condition</th>
<th>Temperature</th>
<th>PH</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 50 mM imidazole, 5 mM sodium β-glycerophosphate (buffer A), 150 mM NaCl, 10 mM MgCl₂</td>
<td>Standard buffer condition</td>
<td>Room温度/4°C</td>
<td>6.5/7.0/7.5</td>
<td>Strong binding of MPR 300, but no binding of MPR 46</td>
</tr>
<tr>
<td>2. Buffer A with 90 mM NaCl, each of 10 mM MgCl₂, MnCl₂, CaCl₂</td>
<td>All the three metal ions included and ionic strength was maintained constant by reducing NaCl to 90 mM</td>
<td>Room温度/4°C</td>
<td>6.5/7.0/7.5</td>
<td>No binding</td>
</tr>
<tr>
<td>3. Buffer A with 150 mM NaCl</td>
<td>In the absence of metal ions</td>
<td>Room温度/4°C</td>
<td>7.0</td>
<td>No binding</td>
</tr>
<tr>
<td>4. Buffer A with increasing concentration of MgCl₂</td>
<td>At an increasing concentration of metal ions</td>
<td>Room温度/4°C</td>
<td>7.0, 7.0, 6.5</td>
<td>No binding</td>
</tr>
<tr>
<td></td>
<td>2 mM MgCl₂, 5 mM MgCl₂, 10 mM MgCl₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Buffer A with 150 mM NaCl, 10 mM MgCl₂</td>
<td>Rebinding of the unbound to a fresh column to see if there is any competitive binding between MPR 300 and MPR 46</td>
<td>Room温度/4°C</td>
<td>6.0/6.5/7.0</td>
<td>No binding</td>
</tr>
<tr>
<td>6. Buffer A with each of 10 mM MgCl₂, MnCl₂, CaCl₂</td>
<td>NaCl completely omitted and all the three metal ions included</td>
<td>Room温度/4°C</td>
<td>6.0/6.5/7.0/7.5</td>
<td>Binding of MPR 46 detected</td>
</tr>
</tbody>
</table>

Table 8: Various buffer conditions used for optimizing the binding conditions of chicken MPR 46 to PM gel (0.2 ml, analytical affinity chromatography)
Analytical PM gel equilibrated with 50 mM imidazole pH 6.0/6.5/7.0, 10 mM MgCl₂, 10 mM MnCl₂, 10 mM CaCl₂ and 5 mM sodium β-glycerophosphate. Each experiment was repeated twice and the mean of two experiments is indicated in the Table 9.

**Table 9:** Mean values of the PM gel binding efficiency of chicken MPR 46 at modified buffer condition at varied pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Flow through</th>
<th>Glucose 6-p Eluate</th>
<th>Mannose 6-p Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>44.5%</td>
<td>1.2%</td>
<td>54.3%</td>
</tr>
<tr>
<td>6.5</td>
<td>31.3%</td>
<td>5.3%</td>
<td>63.4%</td>
</tr>
<tr>
<td>7.0</td>
<td>39.9%</td>
<td>9.45%</td>
<td>50.45%</td>
</tr>
</tbody>
</table>

2.3.4 Identification of Fish MPR 46:

The presence of MPR 46 in fish was identified by metabolic labeling of Xiphophorus fish embryonic cells, extraction of labeled membrane proteins and direct immunoprecipitation with anti-MSC1 antibody. The binding affinity was tested employing similar optimal binding condition standardized for chicken MPR 46 to PM gel (that is addition of 10 mM each of MgCl₂, MnCl₂, CaCl₂ to a modified column buffer pH 6.5 and complete omission of NaCl). The column fractions (unbound, 5 mM glucose 6-phosphate and 5 mM mannose 6-phosphate eluate) were subjected to immunoprecipitation with anti-MSC1 antibody and immunoprecipitates were analyzed on SDS-PAGE and detected by fluorography (Fig 12A). A single band with an
electrophoretic mobility of 32kDa (under reducing condition) was detected upon SDS-PAGE analysis, which is lower than that of the receptor from chicken cells (37-40kDa).

To know, whether this low molecular mass (32kDa) of fish MPR 46 is due to the difference in the polypeptide length or is it due to the differential glycosylation, the immunoprecipitated fish MPR 46 protein was deglycosylated with PNGase F (chicken MPR 46 was used as a control and was processed in a similar way) and analyzed on 10% SDS-PAGE followed by fluorography (Fig 12B). The deglycosylated fish MPR 46 was shown to have electrophoretic mobility of 26 kDa similar to that of deglycosylated chicken and mammalian MPR 46 (26.5-28 kDa; Hoflack and Kornfeld., 1985a). The intracellular immunofluorescence staining of MPR 46 in fish cells with anti-MSC1 antibody and detection by a secondary antibody conjugated to Texas-red showed a typical localization pattern with a perinuclear staining of MPR 46 similar to the staining pattern of MPR 46 in BHK wild type 6 cells that are over expressing human MPR 46 (Fig 13).

2.3.5 Identification of Unio MPR proteins:

Membrane proteins from Unio whole animal tissue acetone powder were extracted as described under methods. The clear membrane extract was passed through PM gel equilibrated with the column buffer, 50 mM imidazole-HCl pH7.0, 150 mM NaCl, 5 mM sodium p-glycerophosphate, 0.05% Triton X-100 and 10 mM MnCl₂. The column was washed and eluted sequentially with 5 mM glucose 6-phosphate and 5 mM mannose 6-phosphate. The mannose 6-phosphate eluate was analyzed by SDS-PAGE, followed by silver staining (Fig 14). A single band with an apparent molecular mass of 300 kDa was seen and MPR 46 protein could not be detected.
**Figure 12:** Identification of MPR 46 in *Xiphophorus* Cells.

(A) A membrane extract of metabolically labeled *Xiphophorus* cells was subjected to PM-Sepharose affinity chromatography and MPR 46 from the column fractions was immunoprecipitated with anti-MSC1 antibody. Conditions are described under methods. The immunoprecipitates were analyzed by 10% SDS-PAGE under reducing (lanes 1-3) and non-reducing (lane 4) conditions followed by fluorography. **Lane 1**, flow through; **lane 2**, glucose 6-phosphate eluate; **lane 3 and 4**, mannose 6-phosphate eluate.

(B) Deglycosylation of fish (lanes 5 and 6) and chicken (lanes 7 and 8) MPR 46 by PNGase F: MPR 46 was isolated by PM-Sepharose chromatography and was immunoprecipitated with anti-MSC1 antibody. The immunoprecipitate was split into two equal aliquots and treated for 16 h with (lanes 6 and 7) or without (lanes 5 and 8) PNGase F followed by 10% SDS-PAGE and fluorography. Arrows: fish MPR 46, asterisk: chicken MPR 46, arrowhead: deglycosylated MPR 46.

**Figure 13:** Intracellular Immunofluorescence Staining for the Localization of MPR 46. Both fish *Xiphophorus* embryonic cells and BHK wild type 6 cells (over expressing human MPR 46) were treated with anti-MSC1 antibody and detected with a secondary antibody which is goat anti-rabbit IgG conjugated to Texas-red. As a negative control both cells were treated in parallel with affinity purified rabbit pre-immune IgG. 1 and 2 fish cells stained with pre-immune and immune IgG respectively, 3 and 4 are BHK wt6 cells treated with pre-immune and immune IgG respectively.
Figure 12

Figure 13

Fish MPR46

BHK-hu.MPR 46
From 100 g of acetone powder 80 μg of purified protein was obtained. For biochemical and immunological characterization, the affinity-purified *Unio* MPR 300 was subjected to radioiodination. Radio iodinated *Unio* MPR 300 was rebound to PM gel and specifically eluted with 5mM mannose 6-phosphate only but could not be eluted with 5 mM glucose 6-phosphate. The electrophoretic mobility shift of *Unio* MPR 300 was found to be similar to that of radio iodinated MPR 300 from goat under reducing and non-reducing conditions (Fig 15). The pH optimum for the binding of *Unio* MPR 300 to PM gel was found to be 6.0 (Tig 16), which is slightly lower than what has been reported for mammalian MPR 300 (Stein et al., 1987a). An antibody raised against *Unio* MPR 300 specifically reacts with the purified iodinated MPR 300 protein and also cross-reacted with goat MPR 300 (Fig 17), suggesting that MPR 300 from invertebrate species and mammals are immunologically related.

2.3.6 Partial Amino acid Sequencing of *Unio* MPR 300:

The affinity purified *Unio* MPR 300 protein was subjected to reductive carboxymethylation, followed by desalting on Sephadex G25 column (Fig 18), the protein peak collected at 2.67 min was used for tryptic digestion. Tryptic peptides were separated on HPLC column (Silica gel overlaid with C₈ alkyl groups) and fractions were collected by the use of automated fraction collecting system. The purity and molecular weight of the tryptic peptides was determined by mass spectrometry. Three peptides (Fig 19 A, B,C) were found to be in pure form and were sequenced by automated Edman’s degradation method. The sequence homology of the tryptic peptides of putative *Unio* MPR 300 to the corresponding sequences of mammalian MPR 300 provided strong evidence that it is the putative MPR 300. The sequence data obtained is shown in the following page
Figure 14: SDS-PAGE Analysis of Affinity Purified *Unio* MPR 300 on PM gel. The membrane protein extract obtained from the whole animal tissue was subjected to affinity chromatography on PM gel in the presence of divalent cations. The mannose 6-phosphate eluate was analyzed on 7.5% polyacrylamide gel. **Lane 1**, Standard high molecular weight markers; **lane 2**, mannose 6-phosphate eluate from PM gel.

Figure 15: Mobility Shift of MPR 300 Under Reducing and Non-reducing SDS-PAGE. Apparent molecular mass of radioiodinated MPR 300 purified from *Unio* whole animal tissue (lanes 1 and 2) and goat liver (lanes 3 and 4) was analyzed on 7.5% polyacrylamide gel under reducing (**lanes 1 and 3**) and non-reducing (**lanes 2 and 4**) conditions.
Figure 16: Effect of pH on the Binding of *Unio* MPR 300 to Immobilized Phosphomannan. Radioiodinated MPR 300 was bound to 0.2 ml of PM gel at varied pH range as described under methods. The column fractions (both flow through and eluted) were subjected to TCA precipitation and both pellets and supernatant were quantitated in a γ-ray counter. Bound MPR 300 was calculated as percentage of TCA precipitable radioactivity in the 5 mM mannose 6-phosphate eluate relative to total TCA precipitable radioactivity recovered in all column fractions.

Figure 17: Immunoreactivity of α-*Unio* MPR 300 antibody with MPR 300 from *Unio* and Goat. The radioiodinated MPR 300 from *Unio* (lane 1 and 2) and goat (lane 3 and 4) was immunoprecipitated with α-*Unio* MPR 300 antiserum and also with the pre immune serum. The immunoprecipitates were analyzed on 10% polyacrylamide gel followed by autoradiography. Lane 1 and 3, MPR 300 immunoprecipitated with preimmune serum; lane 2 and 4, MPR 300 immunoprecipitated with immune serum. Dashed arrow indicates the degraded *Unio* MPR 300.
Figure 18: Gel filtration chromatogram of Unio MPR 300 for the separation of reductive carboxy methylated Unio MPR 300 protein fraction from the salt fraction
Figure 19: Mass Spectrometry of *Unio* MPR 300 Tryptic Peptides. The affinity purified *Unio* MPR 300 was subjected to tryptic digestion and the peptides were separated on reverse phase HPLC. The separated peptides were analyzed by mass spectrometry (thin film method). The three peptides shown in the figure were used for sequencing. (A) Peptide 1; (B) Peptide 2; (C) Peptide 3.
<table>
<thead>
<tr>
<th>Peptide No./Species</th>
<th>Amino acid number corresponding to human MPR 300 total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td></td>
</tr>
<tr>
<td>MPR 300 U</td>
<td>146 156 TLGTPEFV (V/T) AT</td>
</tr>
<tr>
<td>MPR 300 C</td>
<td></td>
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<tr>
<td>MPR 300 B</td>
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<tr>
<td>MPR 300 M</td>
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<tr>
<td>MPR 300 H</td>
<td></td>
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<tr>
<td>Peptide 2</td>
<td>1072 1084 FLHQDIDS(S)LGIR</td>
</tr>
<tr>
<td>MPR300U</td>
<td></td>
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<tr>
<td>MPR300C</td>
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<td>MPR300B</td>
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<td>MPR300M</td>
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<tr>
<td>MPR300H</td>
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<tr>
<td>Peptide 3</td>
<td>1750 1764 VAGP (P/R) I LN (P) IAN ? VY</td>
</tr>
<tr>
<td>MPR 300 U</td>
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<td>MPR 300 C</td>
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<td>MPR 300 M</td>
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<td>MPR 300 H</td>
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*Aminoacids shown in parenthesis are uncertain.

Multiple sequence alignment data of *Unio* (U) MPR 300 tryptic peptides 1, 2, and 3 with that of MPR 300 sequences from chicken (C), bovine (B), mouse (M), and human (H).
2.4 DISCUSSION

With the discovery of two distinct \textit{mannose} 6-phosphate receptors involved in the specific sorting and targeting of lysosomal enzymes to lysosomes in eukaryotic cells, it turned out to be a major interest to investigate, when exactly these receptors took over the mannose 6-phosphate independent pathway which is still conserved to some extent in well established mammalian cells and which is the sole mechanism in the primitive eukaryotes (Heute-Perez et al., 1999).

When the present investigation was started, it was known that MPR proteins do occur in most of the mammals and some of the non-mammalian vertebrates like aves, reptiles and amphibians (Matzner \textit{et al}., 1996; for review see Pohlmann, 1996; Siva kumar \textit{et al}., 1997). The MPR proteins were purified from various species by affinity chromatography using immobilized phosphomannan/ pentamannosylphosphate/ \textit{Dictyostelium discoideum} lysosomal enzyme secretions/ lysosomal enzymes as affinity ligands. We have developed two new simpler affinity matrices by coupling PM and PMP to divinylsulfone activated Sepharose. At first, the efficiency of the matrices was tested by passing the goat liver tissue membrane protein extract over the PM gel and it was found to be as efficient as the earlier methods used for the purification of both receptors (Udaya lakshmi and Siva kumar., 1996).

Exploiting the fact that MPR 46 requires divalent cations for optimal binding to its ligands \textit{in vitro}, a new approach was developed for the separation of the goat MPR proteins. The mixture of goat MPR proteins eluted with 5 mM mannose 6-phosphate from PM gel were dialyzed extensively against 2mM EDTA containing column buffer and passed through PMP gel equilibrated with the same buffer. The separation of the two receptors was well achieved with the new method and is highly reproducible and comparable to the \textit{E-Affigel} that has been used earlier to separate the mixture of the two MPR proteins (Hoflack and Kornfeld., 1985; Stein \textit{et
al., 1981). In summary, the simplified protocol described by us for the separation of the two receptors (Siva kumar and Udaya lakshmi., 1997) avoids the growth of *Dictyostelium discoideum*, collecting lysosomal enzyme secretions and coupling them to Affigel as has been described earlier.

To further extend the knowledge about the occurrence of the receptors down the evolution, we looked for the presence of receptors in the earliest non-mammalian vertebrate, fish and the invertebrate *Unio*. When the mannose 6-phosphate eluates from PM-Sepharose affinity chromatography of detergent extracts obtained either from fish liver tissue or *Unio* whole animal tissue was analyzed by SDS-PAGE, a single band with an apparent molecular mass of 300 kDa, the typical electrophoretic mobility of mammalian MPR 300, was detected and MPR 46 could not be detected. From this data, it can not be predicted whether the concentration of MPR 46 is too low to be detected or whether it is failing to bind the affinity matrix under the conditions used or it may be completely absent.

The authenticity of the 300 kDa protein purified from fish and *Unio* as the MPR protein was established by its ability to bind on phosphomannan gels and specific elution with the 5 mM mannose 6-phosphate only and not with the glucose 6-phosphate. Additionally the mobility shift of the receptor observed under reducing and non-reducing conditions in SDS-PAGE demonstrates the presence of internal disulfide bonds in the receptor which is a characteristic feature of membrane proteins.

It was also observed that antibodies raised against *Unio* MPR 300 cross-reacted with goat MPR 300, suggesting that MPR 300 from invertebrate species (*Unio*) and mammals is immunologically related. It is interesting to note that the receptor from the non-mammalian vertebrates like chicken, garden lizard, frog and fish (present study) and invertebrate *Unio*
showed similar molecular mass and electrophoretic behavior. The optimal pH for binding to phosphomannan was found to be 7.0 for fish MPR 300 and a lower binding in the range of 6.0-7.5 as described earlier for mammalian MPR 300 (Stein et al., 1987a), essentially no binding was observed at pH 5.5 or below. Whereas Unio MPR 300 was shown to exhibit a pH optimum of 6.0, which is slightly lower than what was reported earlier for mammalian MPR 300 (Stein et al., 1987a). Although it is evident from the biochemical and immunological methods that the 300 kDa protein purified from Unio is the putative MPR 300, further evidence supporting this came from the partial amino acid sequencing of the three short tryptic peptides derived from purified Unio MPR 300 that showed 95-98% sequence homology with the MPR 300 sequences already described.

The presence of MPR 46 in fish was investigated by direct immunoprecipitation of MPR 46 from metabolically labeled membrane protein extract of fish cells with anti-MSC1 antibody which was shown to cross-react with non-mammalian MPR 46 (Siva kumar et al., 1997). Preliminary experiments have shown that MPR 46 from non-mammalian cell lines (chicken and fish) did not bind to phosphomannan under the buffer conditions used to purify mammalian receptors (buffer containing 150 mM NaCl) (Stein et al., 1987a). In contrast, MPR 300 from chicken has efficiently bound under these conditions. Addition of 10 mM each of MgCl2, MnCl2, CaCl2 to a modified column buffer with NaCl reduced to 90 mM, to compensate for the increase in osmolarity, did not improve binding of MPR 46 from chicken or fish. Only when NaCl was omitted, MPR 46 from chicken and fish cells efficiently bound in the presence of divalent cations and were specifically eluted with 5 mM mannose 6-phosphate, but not with glucose 6-phosphate.

The electrophoretic mobility of the fish protein eluted from PM-Sepharose (32 kDa under reducing conditions) was found to be lower than that of the receptor from chicken cells (37 kDa-
40 kDa). Despite of the lower molecular mass of the fish receptor, three lines of evidence support the assumption that this fish protein is indeed related to MPR 46 from other vertebrates: first, the 32kDa protein in bound and unbound fractions of PM-Sepharose cross-reacts with the affinity purified anti-MSC1 antibody raised against the conserved cytoplasmic domain of mammalian MPR 46. Second, the reduced and alkylated fish receptor showed a decreased electrophoretic mobility compared to the non-reduced, which indicates that it contains internal disulfide bonds like the mammalian and avian MPR 46 (for review see Hille-Rehfeld., 1995; Matzner et al., 1996). Third, when the fish receptor was deglycosylated by PNGase, its electrophoretic mobility (26 kDa) was similar to that of deglycosylated chicken MPR 46. Taken together, our data suggests that the 32 kDa protein from fish cells represents an under-glycosylated equivalent of avian and mammalian MPR 46 which binds to phosphomannan with low affinity.

The above studies further confirm that both MPRs are consistently present among all vertebrate species (non-mammalian and mammalian) with phosphomannan binding abilities as do the receptors from mammals. But in the invertebrate Unio, only MPR 300 could be detected and it still remains to be investigated whether they also contain MPR 46 homologue. Because of the non-availability of Unio cell line, use of snail cell line (Biomphalaria glabrata., which belongs to the same phylum) for metabolic labeling and immunoprecipitation of the labelled membrane extracts might give valuable information regarding the presence or absence of MPR 46 homologue in these invertebrate species. The structural and functional properties of the non-mammalian vertebrate/ invertebrate MPR proteins purified in this study need to be established.