Identification of MPR dependent targeting of lysosomal enzymes in the invertebrate mollusc (Biomphalaria glabrata) cells
PART A: Characterization of the mannose 6-phosphate receptor (Mr 300 kDa) protein dependent pathway of lysosomal enzyme targeting in Biomphalaria glabrata (Bg) cells

![Diagram](image1)

PART B: Ligand binding properties of cation independent mannose 6-phosphate receptor protein from Biomphalaria glabrata (Bg) cells

![Diagram](image2)
4A.1. INTRODUCTION

The importance of M6P recognition marker on the lysosomal enzyme in higher eukaryotes and the role of specific receptors in their targeting ability among vertebrates (mammalian and non-mammalian) have been described in the preceding chapters. These CIMPR and CDMPR are transmembrane glycoproteins and are conserved from mammals to invertebrates. The M6P receptor dependent targeting mechanism has been described not only in mammals but also in birds and reptiles (Clairmont and Czech, 1989; Siva Kumar et al., 1997; Praveen Kumar and Siva Kumar, 2008). It has been shown earlier that MPR 300 functions in targeting of newly synthesized lysosomal enzymes and also in the endocytosis of exogenous lysosomal enzymes with the use of antibodies that will block the ligand binding site (von Figura et al., 1984; Gartung et al., 1985). The MPR 300 present at the cell surface can endocytose extracellular ligands, endocytosed MPRs can recycle back to the cell surface or alternatively, move via late endosomes to the TGN to sort newly synthesized lysosomal enzymes. The entire cellular pool of MPRs (300 and 46) can cycle between intracellular compartments and cell surface within a period of few hours (Sahagian, 1984). The CIMPR also interacts with M6P containing ligands that do not have hydrolase activity (Dahms and Hancock, 2002). In non-mammalian vertebrates both putative receptors have been identified in the chicken, reptiles, amphibians and fish (Matzner et al., 1996; Siva Kumar et al., 1997; Siva Kumar et al., 1999). In the invertebrates, so far the putative receptors have been affinity purified and biochemically characterized in starfish and in the molluscs viz., unio and Biomphalaria glabrata (Bg) cells (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002). More recently, an extensive study on the star fish MPR 46 protein carried out by us provided the first evidence on its structure function relationship (Sivaramakrishna and Siva Kumar, 2008). In order to gain more insights into the MPR targeting system in the invertebrates, the present study was taken up to understand the role of MPR 300 protein from Bg cells. The unio MPR 300 antiserum used in this study was obtained earlier in the laboratory by injecting purified unio MPR 300 protein in a rabbit. The specificity of the antiserum to recognize unio MPR 300 protein was shown earlier (Udaya Lakshmi et al., 1999). In the present study, the Bg cells were challenged with unio MPR 300 antiserum and the disappearance of CIMPR was followed,
simultaneously the resultant effect on the fate of newly synthesized lysosomal enzymes was also analyzed. The M6P receptor proteins synthesized in cultured chinese hamster ovary cells and human fibroblasts persist with half-lives 6-32 h (Creek and Sly, 1983; Sahagian and Neufeld, 1983). In the present study, the observed half life of MPR 300 receptor protein (CIMPR) from Bg cell was ~ 13 h.

4A.2. MATERIALS AND METHODS

4A.2.1. Materials

Biomphalaria glabrata (Bg) cells used in the present study were kindly provided by Dr. Colette Dissous, Unité 547 INSERM, Institut Pasteur de Lille, 59019 Lille Cedex, France. The Schneider’s Drosophila medium, lactalbumin hydrolysate for cell culture was from Gibco and fetal bovine serum (FBS) from Cambrex, India. Galactose and the chromogenic substrates used for enzyme assays were purchased from Sigma. The following antibodies were available in the laboratory; α-fucosidase antiserum (raised against purified protein from unio), β-hexosaminidase antiserum (raised against purified mouse protein), arylsulfatase A antiserum (raised against human protein), MSC1 antibody (an affinity purified human MPR 46 cytoplasmic tail antibody), unio MPR 300 antiserum (raised against MPR 300 protein purified from unio, whole animal; all antibodies used were polyclonal antibodies unless specified). Lamp-1 monoclonal antibody was kindly provided by Prof. Dr. Stefan Hoening, University of Koeln, Germany. All other chemicals used in the study were of high purity and were purchased locally. All the centrifugation steps in this study were done in Biofuge stratos centrifuge, Heraeus in 1.5 ml rotor unless otherwise mentioned.

4A.2.2. Lysosomal enzymes assays

The Biomphalaria glabrata (Bg) cells were cultured in the complete medium (22 ml of Schneiders Drosophila medium, 450 mg lactalbumin hydrolysate, 130 mg galactose, penicillin/Streptomycin (5 μg/ml), 1-2 mg phenol red, water 68 ml, pH 7.2 and 10% heat inactivated fetal bovine serum) in standard culture flasks at 28°C without CO₂. Confluent cultures are detached from the surface by gushing with PBS (10 mM sodium
phosphate buffer pH 7.4, containing 150 mM NaCl); cell pellet was collected by
centrifugation at 2991×g for 10 min. The pellet was suspended in 0.1 M sodium acetate
buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA,
sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on
ice, and centrifuged in a Beckman centrifuge using a fixed angle 80Ti rotor at
161,280×g for 35 min. The supernatant obtained at this step is referred as acetate
supernatant and was used for enzyme assays. The lysosomal enzyme activities of β-
hexosaminidase, α-galactosidase, α-mannosidase, α-fucosidase were assayed using p-
nitrophenyl derivatives of the respective substrates and arylsulfatase A was assayed
using 4-Nitrocatechol sulfate dipotassium salt as described (page 54, section 3.2.9).
Each of the enzyme activity was done in duplicates in two individual experiments and
the results obtained were plotted using Sigma plot 9.0 taking the averages and standard
deviation for the error bars.

4A.2.3. Metabolic labeling and immunoprecipitation

_Biomphalaria glabrata_ (Bg) cells were cultured in 6 cm petri plates and labeled
with 250 µCi of [35S] methionine for 16 h as described (Siva Kumar and von Figura,
2002). The labeled cells were detached from the surface by gushing with TBS (20 mM
Tris-HCl pH 7.4, 150 mM NaCl). The pellet was suspended in 0.5 ml of TBS containing
1 mM PMSF, 1 µg/ml leupeptin and pepstatin A. The cells were then solubilized by
adding 0.5 ml of 2% Triton x-100, 0.1% NP-40 in TBS (X2 IP buffer) incubated on ice for
20 min and centrifuged at 14006xg for 15 min. To 300 µl of the supernatant obtained,
1% of respective antiserum, 20 µl of protein A-agarose slurry were added, volume finally
made up to 500 µl with 1% Triton x-100, 0.05% NP-40 in TBS (IP buffer), incubated
over night at 4°C with rotation, centrifuged at 3502xg, and the supernatants were
discarded carefully leaving few µl to avoid pipetting protein A-agarose beads.
Resuspended in 500 µl of IP buffer and centrifuged again, the wash steps were
repeated 3-4 times and the final supernatant was pipetted out completely with a narrow
tip. Finally to the moist pellet of beads, 30 µl 2X SDS-PAGE sample buffer (reducing)
was added, boiled at 95°C for 5 min and loaded on to 10% SDS-PAGE. The gel was
then fixed for 30 min in 50% methanol, 10% acetic acid and sequentially passed
through 1, 2 and 3 DMSO (Dimethylsulphoxide) bath for dehydration with 20 min incubation in each. The gel was finally incubated over night in 20% (w/v) PPO (2, 5 diphenyl oxazole) in DMSO; following day PPO on the gel was precipitated with a water wash (the gel turns white). The gel was placed in saran wrap dried and exposed to a Konica X-ray film or phosphorimager.

4A.2.4. Depletion of MPR 300 using unio MPR 300 antiserum

Equal number of Bg cells were seeded in 12 well plates and labeled with 100 µCi of [35S] methionine for 16 h and chased up to 24 h in presence of 1% preimmune serum (control). In a separate experiment it was chased up to 240 min in presence of 1% unio MPR 300 antiserum. The cell lysates were first treated with protein A-agarose and later subjected to immunoprecipitation with unio MPR 300 antiserum as described above. The band intensities of the developed autoradiogram were analyzed using Image J software, plotted using sigma plot 9.0.

4A.2.5.Effect of CIMPR depletion on sorting of endogenous lysosomal enzymes

Bg cells were cultured in 6 cm petri plates and labeled with 250 µCi of [35S] methionine for 24 h in presence of 1% unio MPR 300 antiserum (experimental) or in presence of preimmune serum (control). The lysosomal enzymes present in the control and experimental cells were immunoprecipitated as described above. To detect the secreted lysosomal enzymes, the 35S-labeled medium was centrifuged to remove cell debris; pH adjusted by adding 50 µl of 1M Tris pH 8.0. From this 400 µl each was subjected to immunoprecipitation as done for the cell lysate except that TBS was used in place of IP buffer and samples analyzed by SDS-PAGE. The gels processed as described above, the PPO treated and dried gels were exposed to phosphorimager.

4A.2.6. Internalization of labeled exogenous lysosomal enzymes

Confluent cultures in 75-cm² flasks were incubated for 24 h in the labeling medium supplemented with protease inhibitors (aprotinin and leupeptin each 5 µg/ml concentration), 250 µCi of [35S] methionine, 10 mM NH₄Cl (to induce the secretion of lysosomal enzyme precursors) (Hasilik and Neufeld, 1980). The secreted enzymes
were precipitated by adding 2.5 g of \((\text{NH}_4)_2\text{SO}_4\), after stirring for 2 h at 4°C it was centrifuged in 8 x 50 ml fixed angle rotor at 17226xg, 20 min. The pellet obtained was dissolved in 1 ml of sterile water and dialyzed extensively against \(Bg\) cell medium without serum. To this dialyzed sample 0.25 ml of fetal bovine serum was added and the volume was adjusted to 5 ml with \(Bg\) medium, sterile filtered through 0.22 µm disposable syringe filter. These labeled secretions were offered to a confluent 75-cm² culture flask which was incubated previously with MS medium (350 mg NaCl, 16 mg KCl, 9 mg Na₂HPO₄ 2H₂O, 45 mg MgSO₄ 7H₂O, 53 mg CaCl₂ 2H₂O, 5 mg NaHCO₃, 150 mg D-glucose, D-150 mg trehalose, dissolved in 100 ml of autoclaved water and sterile filtered) containing protease inhibitors for 24 h. At the end of additional 24 h incubation in the presence of labeled secretions, cells were detached and the cell lysates were subjected to immunoprecipitation for detecting internalized lysosomal enzymes as described above, followed by SDS-PAGE under reducing conditions, the protein bands were identified by fluorography and exposure to phosphorimager.

4A.2.7. **Immuofluorescence studies**

The cells were immunostained with unio MPR 300 antiserum, Lamp-1 antibody, and in a separate experiment with MSC1 antibody and Lamp-1 as described (page 54, section 3.2.10).

4A.3. **RESULTS**

4A.3.1. **Enzyme assays and immunoprecipitation of lysosomal enzymes**

To detect the lysosomal enzyme activities in \(Bg\) cells, acetate supernatant from the cells was assayed using chromogenic substrates. Of the five lysosomal enzymes assayed viz., \(\beta\)-hexosaminidase, \(\alpha\)-galactosidase, \(\alpha\)-mannosidase, \(\alpha\)-fucosidase and arylsulfatase A, arylsulfatase A showed very high enzyme activity for the same amount of protein taken (Fig 4A.4.1). In order to identify the respective lysosomal enzymes, \(Biomphalaria glabrata\) (\(Bg\)) cells were metabolically labeled with 250 µCi of \([^{35}\text{S}]\) methionine for 16 h and subjected to immunoprecipitation using the respective enzyme antiserum. The gels were treated for fluorography and developed by autoradiography.
Arylsulfatase A showed both precursor and mature forms (Fig 4A.4.2A). β-hexosaminidase showed two bands, a precursor form and a mature form below it (Fig 4A.4.2B). α-fucosidase showed a single band (Fig 4A.4.2C). The receptor proteins were also identified by using the *unio* MPR 300 antiserum and MSC1 antibody for detecting MPR 300 (Fig 4A.4.2D) and MPR 46 (Fig 4A.4.2E) proteins, respectively. An additional band seen above the MPR 46 protein in Fig 4A.4.2E could be a cross-reactive material or an aggregate form of the receptor.

4A.3.2. Effect of *unio* MPR 300 antiserum on MPR 300 and endogenous lysosomal enzymes

Addition of 1% *unio* MPR 300 antiserum to the cultured *Biomphalaria glabrata* (Bg) cells resulted in the clearance of MPR 300 protein. The membrane bound MPR 300 was immunoprecipitated from the cells that were labeled with [35S] methionine for 16 h and chased up to 24 h in the presence of preimmune serum or up to 240 min in the presence of *unio* MPR 300 antiserum, as the case may be. The lysates were first treated with protein A-agarose beads to deplete the receptor antibody complexes that might have formed during the incubation period with antiserum and the supernatants were used for immunoprecipitation. The level of the MPR 300 protein was followed for 0, 4, 8, 12, 16 and 24 h in preimmune serum treated cells and followed for 0, 20, 40, 60, 120, 240 min in antiserum treated cells. The fluorography treated gels were dried and developed by autoradiography, the signal intensities in each lane was calculated by image J software. Graphs were plotted in Sigma plot 9.0 taking time on X-axis and % of the receptor protein compared with the 0 h sample on Y-axis. In control cells that were incubated with 1% of preimmune serum, the MPR 300 protein disappeared with a half life of ~ 13 h (Fig 4A.4.3A). In case of antiserum treated cells the receptor half life was dramatically reduced to ~ 20 min (Fig 4A.4.3B). Exposure to antibody induces the degradation of receptors or accumulation of receptor-antibody complexes within the cells (von Figura et al., 1984).

The effect of antiserum on missorting of endogenous lysosomal enzymes was analyzed in the cells that were metabolically labeled with [35S] methionine for 24 h in the presence of preimmune serum and *unio* MPR 300 antiserum. After 24 h, both the cell
lysate and the medium from preimmune treated, \textit{unio} MPR 300 antiserum treated were analyzed for the presence of arylsulfatase A (Fig 4A.4.4A), \( \beta \)-hexosaminidase (Fig 4A.4.4B) and \( \alpha \)-fucosidase (Fig 4A.4.4C). From the results obtained it is evident that 80-85% of the tested enzymes were secreted into the medium treated with \textit{unio} MPR 300 antiserum compared to the preimmune serum treated sample. The preimmune serum treated cells retained significant amounts of the enzymes but in the \textit{unio} MPR 300 antiserum treated cells due to the depletion of the MPR 300 protein; the enzymes were secreted into the culture medium.

\textbf{4A.3.3. Internalization of lysosomal enzymes and confocal microscopy}

75-cm\(^2\) confluent culture flasks were incubated for 24 h in the labeling medium containing 10 mM NH\(_4\)Cl which will induce the secretion of lysosomal enzyme precursors. These labeled secretions enriched in lysosomal enzyme precursors were concentrated by ammonium sulphate precipitation, dialyzed against \(Bg\) medium and offered to another 75-cm\(^2\) culture flasks. Internalized arylsulfatase A (Fig 4A.4.5A), \( \beta \)-hexosaminidase (Fig 4A.4.5B) and \( \alpha \)-fucosidase (Fig 4A.4.5C) from the cells that were incubated for 24 h with the labeled secretions was analyzed by immunoprecipitation. The detection of the respective enzymes reflects the rapid endocytosis of lysosomal enzymes by MPR 300 receptor protein present at the cell surface. The presence of mature forms in a large number of cases tested indicates that these have been processed in the destined lysosomes inside the internalized cells.

The \(Bg\) cells were also studied for the presence and distribution of MPR 300 (Fig 4A.4.6 top panel) and MPR 46 (Fig 4A.4.6 bottom panel) receptor proteins by immunofluorescence. Lamp-1 (Lysosomal associated membrane protein1, a transmembrane protein enriched in late endosomes and lysosomes) was used as a marker protein. Endosomal distribution and partial colocalization of the receptor proteins with Lamp-1 can be seen.
Figure 4A.4.1: The Enzyme activities from the Bg cell acetate supernatant with the respective chromogenic substrates. H, G, M, A and F represent β-hexosaminidase, α-galactosidase, α-mannosidase, arylsulfatase A and α-fucosidase enzyme activities, respectively. Plotted using Sigma plot 9.0 taking the averages of column values and standard deviation for the error bars.
Figure 4A.4.1

Enzymes

Activity in IU/mg of protein

H M FAG

Figure 4A.4.1
Figure 4A.4.2: Immunoprecipitation (IP) of lysosomal enzymes and mannose 6-phosphate receptor proteins from $[^{35}\text{S}]$ methionine labeled $Bg$ cells with 250 µCi for 16 h, gels after fluorography and drying developed by autoradiography or exposed to phosphorimager. (A) IP with arylsulfatase A antiserum, both the precursor and mature forms are indicated by arrows; (B) IP with $\beta$-hexosaminidase antiserum, '+' in the figure represents the precursor form of the enzyme; (C) IP with $\alpha$-fucosidase antiserum, only one from is observed indicated by the arrow; (D) IP with unio MPR 300 antiserum; (E) IP with MSC1 antibody, arrow indicates the receptor protein, band indicated by '::*' could be due to a cross-reactive material.
**Figure 4A.4.3: Depletion of MPR 300 protein.** *Bg* cells pulsed with $[^{35}\text{S}]$ methionine for 16 h and chased for MPR 300 protein using *unio* MPR 300 antiserum by IP. **(A)** chase up to 24 h from *Bg* cells treated with 1% preimmuniserum, the graph plotted below by taking the band intensities shows the disappearance of MPR 300 with a half life of ~13 h. **(B)** chase up to 240 min from *Bg* cells treated with 1% antiserum, the graph plotted below by taking the band intensities shows the drastic loss of MPR 300 with a half life of ~20 min.
Figure 4A.4.3

A  
Pre-Immune serum treated

B  
Antiserum treated

Time in h  
0 4 8 12 16 24

Time in min  
0 20 40 60 120 240

MPR 300 (% of initial)
Figure 4A.4.4: Fate of lysosomal enzymes after treatment with unio MPR 300 antiserum for 24 h. IP of, arylsulfatase A (A), β-hexosaminidase (B), (P and M in the figure represents the precursor and mature forms, respectively) and α-fucosidase (C), present in the cell lysate and in the medium from both the preimmuneserum and antiserum treated Bg cells. In Fig B, it can be seen that in antiserum treated cells mostly the precursor form of β-hexosaminidase is seen secreted in to the medium. The absence of functional MPR 300 resulted in the secretion of lysosomal enzymes in to the medium.
Figure 4A.4.4
**Figure 4A.4.5: Internalization of labeled lysosomal enzyme precursors.** The *Bg* cell lysate was analyzed for the presence of internalized labeled enzymes after an incubation of 24 h. Detected by IP with respective antiserum, arylsulfatase A (A), β-hexosaminidase (B) P in the figure represents the precursor form, and α-fucosidase (C).
Figure 4A.4.5

Arylsulfatase A

β-Hexosaminidase

Mature

P

P

α-Fucosidase
Figure 4A.4.6: Localization of MPR 300 and MPR 46 protein; (A) and (E) incubation with *unio* MPR 300 antiserum and MSC1 antibody, respectively followed by FITC (green) conjugated secondary antibody. (B) and (F) incubation with Lamp-1 antibody followed by Cy3 (red) conjugated secondary antibody. (C) merged image of A and B, (G) merged image of E and F. D and H are the corresponding transmission images. Bar in the top panel is 15 µm and the bottom panel is 23.18 µm.
Figure 4A.4.6
4A.5. DISCUSSION

We have earlier demonstrated the presence of MPR 46 and MPR 300 proteins in unio tissue as well in Bg cells by metabolic labeling and immunoprecipitation with MSC1 antibody and unio MPR 300 antiserum, respectively (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002). Both the receptor proteins were purified to homogeneity by phosphomannan (PM) affinity chromatography, the study also confirmed the mannose 6-phosphate (M6P) dependent binding to PM gel. In this study we focused on the functional aspect of the receptor protein (MPR 300), which is to bind the bulk of lysosomal enzymes in a M6P dependent manner and target them to lysosomes. The MPR 300 protein also binds the extracellular ligands and internalizes them routing to lysosomes. We have identified lysosomal enzymes in Bg cells by enzyme assays and by immunoprecipitation of the $[^{35}S]$ methionine labeled cellular proteins. Due to the limited availability of antibodies in our laboratory, we concentrated only on three lysosomal enzymes viz., arylsulfatase A, β-hexosaminidase and α-fucosidase. The immunoprecipitated samples showed the existence of both the precursor and the mature forms. The antibody reactivity and presence of the receptors were confirmed by immunoprecipitation of MPR 300 and MPR 46 proteins using unio MPR 300 antiserum and MSC1 antibody, respectively. Our aim is to deplete the MPR 300 protein from Bg cells with the use of antiserum and monitor the relative distribution of lysosomal enzymes between cell lysate and medium. The MPR 300 receptor depletion was confirmed by its disappearance from the membranes with a half life of ~ 20 min (Fig 4A.4.3B). The antibodies present in the medium bind to MPR 300 present at the cell surface and makes it inactive further leading to internalization. This receptor-antibody complex upon internalization will lead to its degradation in lysosomes (these complexes that might have formed will escape the detection as the lysates were initially treated with protein A-agarose before immunoprecipitation). The dramatic loss of the receptor indicates that the cell surface receptor will always be in rapid equilibrium with that of intracellular receptor population. The loss of intracellular receptor explains the failure in sorting of newly synthesized lysosomal enzymes, and are hence secreted into the medium (about 80-85%), which are exclusively the precursor forms. The presence of 15-20% of enzymes in the cells treated with MPR 300 antiserum can be attributed to
MPR 46 function. The conversion of larger precursor forms into smaller mature forms is thought to occur after their delivery to lysosomes (Hasilik and Neufeld, 1980; Waheed et al., 1982). In the preimmune serum treated cells very less or no enzyme was detected in the medium conversely bulk of the enzymes was seen intracellularly, and this observation is opposite in case of the antiserum treated cells. This establishes the functional role of the MPR 300 receptor protein in sorting of newly synthesized lysosomal enzymes. To further our knowledge on the endocytic function of the MPR 300 protein, we have incubated the Bg cells with NH₄Cl induced secretions from a different experiment with Bg cells. We then identified the internalized arylsulfatase A, β-hexosaminidase and α-fucosidase enzymes by immunoprecipitation. This suggests and signifies the importance of the cell surface MPR 300 in these cell types which is being able to internalize lysosomal enzymes and target them to the lysosomes. In a separate study we have seen the inhibition of uptake of radioiodinated β-galactosidase (from bovine) and α-fucosidase (from unio) in the presence of unio MPR 300 antiserum (data presented in continuation of this chapter PART-4B), which supports that the uptake of lysosomal enzymes is MPR 300 protein dependent. The immunofluorescence study shows the distribution of the receptor proteins to the endocytic structures. Lamp-1 is used as counter stain, only partial colocalization is observed as Lamp-1 is present on the late endosomes and lysosomes where the receptor proteins are present in very low amounts. The receptor protein and its bound lysosomal enzymes will dissociate in the early lysosomal compartment due to the acidic nature and recycle back to the trans-golgi network (TGN) or move to the plasma membrane to involve in endocytic process (Kornfeld, 1992).

The role of the receptors in lysosomal enzyme targeting in the invertebrates is poorly understood. Only recently we have cloned the gene for the MPR 46 protein in the highly evolved invertebrate starfish and provided the first biochemical evidence to show that this receptor is involved in the lysosomal enzyme targeting, although no such studies have been done for the MPR 300 protein so far (Sivaramakrishna and Siva Kumar, 2008). The present study is an extension in this prospect, to further understand the lysosomal enzyme targeting system in the invertebrate systems. Since we have already identified and characterized the proteins in the molluscs, (unio and Bg cells), we
chose \textit{Bg} cells to study the function of the MPR 300 protein. In summary this is the first report detailing the role of MPR 300 protein from \textit{Bg} cells in segregating newly synthesized lysosomal enzymes and in the endocytosis of extracellular ligands. The findings support our earlier results and hypothesis that the MPR targeting system is an ancient pathway in evolution and it is conserved from molluscs to mammals. Below the molluscs, in annelids and arthropods we have observed only MPR 300 like proteins (Raju et al., 2001). In the \textit{Drosophila melanogaster} lysosomal enzyme receptor protein (LERP) could be identified. Although this protein shows homology to the human MPR 300 to the extent of \(\sim 20\%\), it failed to bind the multivalent PM gel (Dennes et al., 2005), a characteristic feature exhibited by the MPR 300 protein from molluscs to mammals. It still needs to be established whether any other targeting system predates the existing well known trafficking system in the lower organisms below the molluscs in the animal kingdom.

In continuation of these findings, in our next section PART-4B, we studied the binding kinetics of MPR 300 from \textit{Bg} cells towards \(\beta\)-galactosidase (from bovine) and \(\alpha\)-fucosidase (from \textit{unio}) in the presence and absence of specific and non-specific ligands.
Ligand binding properties of cation independent mannose 6-phosphate receptor protein from *Biomphalaria glabrata* (*Bg*) cells
4B.1. INTRODUCTION

Previous studies on ligand binding properties of bovine MPRs revealed that the MPR 300 binds 2 mol, while the MPR 46 binds 1 mol, of M6P per polypeptide. However, since the MPR 46 exists as a dimer, the functional form of MPR 46, like that of MPR 300, contains two M6P binding sites (Tong et al., 1989; Tong and Kornfeld, 1989). From the scatchard plot analysis they also showed that MPR 300 monomer binds 0.9 mol of β-galactosidase with a $K_d$ of 20 nM. Braulke et al. (1987) studied the ability of human fibroblasts to bind and endocytose bovine serum albumin conjugated with about 30 molecules of pentamannose phosphate. The apparent $K_d$ values at 0ºC and 37ºC were 0.5 and 3 nM, while the $K_{uptake}$ was 7 nM. Murray and Neville (1980), reported that low density lipoprotein containing 40-50 pentamannose phosphate bound to receptor with a $K_d$ of <2 nM.

In our earlier studies, to understand the MPR targeting system in the invertebrate molluscs, we have identified putative receptors and purified them to homogeneity by affinity chromatography on multivalent phosphomannan gel (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002). Further we studied the role of MPR 300 on $Bg$ cells in targeting of newly synthesized lysosomal enzymes and also in the internalization of exogenous ligands by depleting the receptor protein from the membranes using specific antiserum, that has been discussed in the preceding section. To conclusively establish the binding specificity and to know the binding parameters, the present study was taken up. In this study, we report the binding properties of MPR 300 protein from $Bg$ cells using β-galactosidase (from bovine) and compare them with α-fucosidase (obtained from the same species unio), in terms of its $K_d$ values. The importance of M6P moiety for binding and internalization of lysosomal enzymes was shown by the use of specific and non-specific ligands. The role of high mannose oligosaccharides present on β-galactosidase and α-fucosidase in binding and internalization was demonstrated by using dephosphorylated and deglycosylated forms of the enzymes. The internalization and sub-cellular distribution of the FITC conjugated α-fucosidase was also studied by confocal microscopy.
4B.2. MATERIALS AND METHODS

4B.2.1. Materials

*Biomphalaria glabrata (Bg)* cells used in the present study were kindly provided by Dr. Colette Dissous, Unité 547 INSERM, Institut Pasteur de Lille, 59019 Lille Cedex, France. β-galactosidase purified from bovine liver, the enzymes ALP, PNGaseF and EndoH, M6P (mannose 6-phosphate), G6P (glucose 6-phosphate) and D-mannose were purchased from Sigma. The Schneider’s Drosophila medium and lactalbumin hydrolysate for cell culture were purchased from Gibco. [125I] was procured from Suyog diagnostics, India. The following antibodies were available in the laboratory, *unio* MPR 300 antiserum (raised against MPR 300 protein purified from *unio*, whole animal) and α-fucosidase antiserum (raised against purified protein from *unio*).

4B.2.2. Radioiodination of β-galactosidase and α-fucosidase

The α-fucosidase used in the present study was purified from *unio* whole animal as described (Siva Kumar et al., 2004). About 50 µg of the respective protein was radioiodinated as below.

Protein to be radioiodinated was dissolved in 20 mM borate buffer pH 8.0 in a conical bottom glass centrifuge tube (borosil). To this 2 µl (200 µCi) of [125I] was added under the hood, the tube was closed with parafilm and incubated on ice for 5 min. The contents were then transferred to IODO-gen tube prepared by uniformly coating 50-80 µl of iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglucoluril, dissolved in dichloromethane to give a final concentration of 1 mM) solution under Nitrogen atmosphere at the bottom of a conical bottom glass centrifuge tube and incubated with rotation on ice for 8 min. The reaction was stopped by transferring the contents to a third conical bottom glass centrifuge tube containing 50 µl of tyrosine (10 mg/ml concentration). The second tube was rinsed with 140 µl of Tris-HCl buffer pH 7.4, containing 1mg/ml BSA, 150 mM NaCl (column buffer) and transferred to third tube. Additional 300 µl of column buffer was added to tube 3 to make up the volume to 500 µl. The iodinated protein fraction was separated from the free iodine by passing the sample over a 10 ml G-25 gel filtration column pre-equilibrated with 20 ml of column buffer. The sample was applied on the gel
and about 12 fractions each of 0.5 ml were collected in 1.5 ml centrifuge tubes placed in the lead aluminum metal holder. 2 µl from each of the tube was aliquoted aside and counted for 1 min in a γ-ray counter.

4B.2.3. Binding of [125I] β-galactosidase

The [125I] β-galactosidase (~ 3 µCi/µg) was taken in a final concentration ranging from 5-50 nM. Bg cells grown to 80-85% confluency in 12 well culture plates were incubated with 10 different concentrations of [125I] β-galactosidase for 90 min at 4°C in binding buffer (Tris-HCl buffer pH 7.4, containing 0.1% BSA, 150 mM NaCl and 5 mM CaCl2). In a separate experiment non-specific binding was determined in the presence of 2 µM non-radioactive β-galactosidase. After incubation, the cells were washed with PBS (10 mM sodium phosphate buffer pH 7.4, containing 150 mM NaCl) containing 1% BSA five times, lysed with lysis buffer (50 mM Tis-HCl buffer pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 0.02% NaN3 supplemented with PMSF, 1 mM EDTA and protease inhibitors) on ice for 30 min. The cell suspension was centrifuged at 2991xg for 2 min. The supernatant was discarded and pellets were counted in a gamma counter, and the amount of bound [125I] β-galactosidase was calculated and normalized to the concentration of the membrane protein. Analysis was performed in duplicates and the average values were presented. Saturation and scatchard plot analysis were carried out using the details in (www.graphpad.com)

4B.2.4. Binding of [125I] α-fucosidase

The [125I] α-fucosidase (~ 3 µCi/µg) was incubated with Bg cells taken in 10 different concentrations ranging from 5-50 nM with 5 nM increment and the experiment was performed as done above for β-galactosidase.

4B.2.5. Treatment of [125I] β-galactosidase and [125I] α-fucosidase with Endo H, PNGase F and alkaline phosphatase

For treatment with Endo H and PNGase F glycosidases, [125I] β-galactosidase and [125I] α-fucosidase (5, 00,000 cpm each) was denatured by boiling with SDS (Endo
H treatment) and 2 µl of NP-40 (PNGase F treatment) incubated for 12 h at 37°C in incubation buffer according to manufactures protocol containing 10 micro units/µl Endo H and 20 micro units/µl PNGase F (Sigma) (pH 7.0). For phosphatase treatment[^125I] β-galactosidase and[^125I] α-fucosidase was incubated in 0.15 M NaCl and 0.01 M Tris-HCl buffer pH 8.0, 50 milli units/µl alkaline phosphatase (Sigma), for 1 h at 37°C, and then diluted to 1 ml with Bg medium. Effect of these treatments on endocytosis and binding was studied.

4B.2.6. Effect of deglycosylation on binding of[^125I] β-galactosidase and[^125I] α-fucosidase to MPRs

The Bg cell membrane extract (about 35-40 µg) was heated at 37°C for 5 min in non-reducing sample buffer, run on 10% SDS-PAGE and transferred to nitrocellulose (NC) membrane. The membrane was cut into individual lanes and incubated for 2 h in 50 mM imidazole, pH 6.5, 150 mM NaCl, 0.05% (v/v) Triton X-100, and 5 mM β-glycerophosphate containing 5% non-fat milk powder (buffer A). The membrane was next incubated in the same buffer containing 2,00,000 cpm/ml of[^125I] β-galactosidase or[^125I] α-fucosidase, untreated or glycosidases treated, as the case may be for 4 h. Subsequently, the membranes were washed 4-5 times for 5 min each with buffer A and finally 3 times with buffer A without milk powder. The membranes were air dried and autoradiographs developed by exposing to Kodak X-ray film for 4-5 days at -70°C.

4B.2.7. Endocytosis of[^125I] β-galactosidase

The endocytosis of[^125I] β-galactosidase by Bg cells was carried out in the absence or presence of M6P (5 mM), G6P (5 mM), D-mannose (5 mM), unin MPR 300 antiserum (1%) and preimmune serum (1%). Bg cells were grown to 80-85% confluency in 6 well culture plates. The cells were rinsed with Bg medium containing 20 mM HEPES pH 7.4 to remove the residual medium. The cells were then incubated with ligands mentioned above for 30 min at 28°C in 1 ml of serum-free medium. At the end of additional 30 min incubation with[^125I] β-galactosidase (2, 00,000 cpm), the cells were washed six times with serum-free medium, three times with Bg medium containing 1 mg/ml bovine serum albumin and five times with PBS. The volume of each wash was 1
ml. After washings the cells were detached from the surface by gushing with TBS (20 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl), cell pellet was collected by centrifugation at 2991×g for 10 min, lysed in about 50 µl of TBS containing 150 mM NaCl, 1 mM PMSF, 1 µg/ml leupeptin, 1% Triton x-100, 0.05% NP-40. Radioactivity in the lysate was measured in a gamma counter and later applied on a 10% SDS-PAGE, and the gel processed for autoradiography. The importance of glycosylation on the radioiodinated enzyme was studied by incubating the Bg cells with ALP, PNGaseF and EndoH treated [125I] β-galactosidase and followed its internalization. A bar graph was plotted in terms of the % of counts compared with the incubation of [125I] β-galactosidase alone taking average of counts in cpm done in triplicates.

4B.2.8. Endocytosis of [125I] α-fucosidase

The endocytosis of [125I] α-fucosidase by Bg cells was carried out in the absence or presence of various ligands as mentioned above and proceeded as done for [125I] β-galactosidase.

4B.2.9. Coimmunoprecipitation of [125I] β-galactosidase and [125I] α-fucosidase with MPR 300

Membrane extraction from the Bg cells was done as described (page 53, section 2.2.4), to this 2, 00,000 cpm of [125I] β-galactosidase or [125I] α-fucosidase, was added as the case may be and made up to 1 ml by adding 20 mM Hepes pH 7.4 buffer containing 300 mM NaCl, 4 mM MgCl2, 3 mM CaCl2, 1 mM PMSF, 2 µg/ml leupeptin. This was kept for rotation for overnight at 4°C, and the receptors and bound 125I ligands were immunoprecipitated with unio MPR 300 antiserum prebound to protein A-agarose beads. The beads were washed with sodium phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST) 6-7 times and the bound [125I] β-galactosidase or [125I] α-fucosidase as the case may be, was specifically eluted with 5 mM M6P by incubating for 1 h at 4°C. In a separate experiment 5 mM G6P and 5 mM D-mannose (non-specific ligands) were used in the elution step. The eluates were run on a 10% SDS-PAGE under reducing conditions and protein bands detected by autoradiography.
4B.2.10. Confocal microscopy

About 5 mg of α-fucosidase was incubated with 550 µl borate buffer (50 mM, pH 9.0) and 100 µl fluorochrome solution of fluorescein-isothiocyanate (5 mg FITC dissolved in one ml of DMSO) overnight at 4°C. Free FITC was removed by desalting using a Sephadex G-25 gel. The endocytosis of FITC conjugated α-fucosidase was studied by incubating with Bg cells grown on cover slips at 28°C. Following incubation of about 30 min the cells were fixed in 4% paraformaldehyde, washed, permeabilised with 0.2% Triton X-100, after blocking, cells were incubated with unio MPR 300 antiserum and detected using Cy5 labeled secondary antibody (Praveen Kumar and Siva Kumar, 2008). In a separate experiment the cells were pre-incubated for 30 min with 5 mM M6P before adding FITC conjugated α-fucosidase.

4B.3. RESULTS

4B.3.1. Determination of Kd values for [125I] β-galactosidase and [125I] α-fucosidase

The Bg cells were incubated with [125I] β-galactosidase or [125I] α-fucosidase in the concentration range of 5-50 nM with 5 nM increment. In a separate experiment the non-specific binding was determined in the presence of 2 µM non-radioactive β-galactosidase or α-fucosidase, as the case may be. The specific binding was taken by subtracting the non-specific counts from the total counts with each of the concentration plotted. The saturation and the scatchard plot analysis was done using graphpad prism 5. The saturation curve for β-galactosidase (Fig 4B.4.1 inset) was obtained by plotting free [125I] β-galactosidase (in nM) on X-axis and specifically bound [125I] β-galactosidase (in nM) on Y-axis, similarly followed for the α-fucosidase (Fig 4B.4.2 inset). From the scatchard plot, the Bmax and Kd values for β-galactosidase are 0.1625 nM, 22.52 nM (Fig 4B.4.1) and 0.1294 nM, 8.090 nM (Fig 4B.4.2) for α-fucosidase. From the Kd values it is clearly evident that α-fucosidase has more affinity towards MPR 300 compared to β-galactosidase.
4B.3.2. Effect of enzymatic deglycosylation on endocytosis and binding of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase by MPRs

The dephosphorylated and deglycosylated form of the enzyme was not internalized by the $Bg$ cells following incubation, and upon loading the lysate no bands were seen in the autoradiogram (Fig 4B.4.4C lanes 7 to 9). In ligand blotting (Fig 4B.4.3) analysis using the ALP, PNGaseF and EndoH treated $[^{125}\text{I}]\beta$-galactosidase or $[^{125}\text{I}]\alpha$-fucosidase (as the case may be) as a probe failed to bind MPR proteins present on NC membrane. The untreated radioiodinated enzyme showed binding towards the MPR proteins (Fig 4B.4.3A and E for the $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase, respectively), the two bands developed are due to its binding to MPR 300 (top) and MPR 46 (bottom). The treated radioiodinated enzymes failed to bind both the MPR 300 and MPR 46 proteins (Fig 4B.4.3B to D and 4B.4.3F to H).

4B.3.3. Endocytosis of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase by MPR 300 is M6P dependent

The endocytosis of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase by $Bg$ cells was carried out separately in the presence of 5 mM M6P, 5 mM G6P, 5 mM D-mannose, unio MPR 300 antiserum (1%) and preimmune serum (1%). After an incubation of 30 min with iodinated enzyme in the presence of these ligands, the cells were lysed and radioactivity was counted, simultaneously the lysate was applied on to 10% SDS-PAGE for visualization of the internalized radioactive enzyme (Fig 4B.4.4C). The extent of inhibition showed by each of them was represented by a bar graph, Fig 4B.4.4A for $\beta$-galactosidase and Fig 4B.4.4B for $\alpha$-fucosidase. The M6P significantly inhibited the uptake of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase by about 95% and no band was observed in the autoradiogram developed (Fig. 4B.4.4C lane 3). Where as G6P and D-mannose showed minimum inhibition between 12-18% and the internalized radioiodinated enzyme can be seen (Fig 4B.4.4C lanes 2 and 4, respectively). Presence of unio MPR 300 antiserum (1%) abolished the endocytosis (93-94%) of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase, preimmune serum (1%) was used as a control which showed minimum inhibition between 10-11%.
4B.3.4. Coimmunoprecipitation of MPR 300 and \(^{125}\text{I}\) \(\beta\)-galactosidase or \(^{125}\text{I}\) \(\alpha\)-fucosidase

The membrane extract of \(Bg\) cells was incubated with 2,00,000 cpm of \(^{125}\text{I}\) \(\beta\)-galactosidase or \(^{125}\text{I}\) \(\alpha\)-fucosidase, as the case may be. The MPR 300 protein and bound radioiodinated enzyme complex was pulled down with \textit{unio} MPR 300 antiserum prebound to protein A-agarose beads. In the elution step, the bound radioiodinated enzyme from the complex could be eluted specifically with 5 mM M6P (Fig 4B.4.5 lane 2) but not with either 5 mM G6P (Fig 4B.4.5 lane 1) or 5 mM D-mannose (Fig 4B.4.5 lane 3).

4B.3.5. Localization of endocytosed FITC-conjugated \(\alpha\)-fucosidase

The endocytic function of the \(Bg\) cell MPR 300 protein was also revealed by confocal microscopy with the use of FITC-conjugated \(\alpha\)-fucosidase. The endocytosed FITC-\(\alpha\)-fucosidase can be seen in Fig 4B.4.6A (green). In the same cells, MPR 300 protein was also visualized with the use of \textit{unio} MPR 300 antiserum and Cy5 labeled secondary antibody (Fig 4B.4.6B). Extensive colocalization of endocytosed FITC-\(\alpha\)-fucosidase with the MPR 300 protein can be seen (Fig 4B.4.6C). The pre-incubation of \(Bg\) cells with 5 mM M6P resulted in failure of FITC-\(\alpha\)-fucosidase uptake and the green fluorescence was not observed (Fig 4B.4.6E), the same cells were stained for the presence of MPR 300 protein (Fig 4B.4.6F).
FIGURES 4B.4
Figure 4B.4.1: Saturation binding assay of $^{125}\text{I}$ β-galactosidase on $B_g$ cells. The $B_g$ cells were incubated with 10 different concentrations (5-50 nM) of the enzyme in a 12 well plate. The non-specific binding was determined by incubating the $^{125}\text{I}$ β-galactosidase in presence of 2 µM non-radioactive β-galactosidase. The inset graph is the saturation curve plotted by taking free (on X-axis) verses specifically bound (on Y-axis) $^{125}\text{I}$ β-galactosidase, and the scatchard plot analysis gave a $K_d$ value of 22.52 nM. Graphs were plotted by graphpad prism 5.
Figure 4B.4.2: Saturation binding assay of $[^{125}\text{I}]$ α-fucosidase on Bg cells. The Bg cells were incubated with 10 different concentrations (5-50 nM) of the enzyme in a 12 well plate. The non-specific binding was determined by incubating the $[^{125}\text{I}]$ α-fucosidase in presence of 2 µM non-radioactive α-fucosidase. The graphs were plotted using graphpad prism 5. The inset is the saturation curve plotted by taking free (on X-axis) versus specifically bound (on Y-axis) $[^{125}\text{I}]$ α-fucosidase, and the scatchard plot analysis taking bound (on X-axis) versus ratio of bound and free (on Y-axis) gave a $K_d$ value of 8.090 nM.
Figure 4B.4.2

Scatchard plot

Bound/Free

Bound

Figure 4B.4.2
**Figure 4B.4.3: Ligand blotting:** About 35-40 µg of Bg cell membrane extract was separated on 10% SDS-PAGE and transferred to NC membrane. The cut lanes are incubated with 2,00,000 cpm/ml of [¹²⁵I] β-galactosidase; (A) normal, untreated. (B) ALP, alkaline phosphatase treated. (C) PNGase F, peptidyl N-glycosidase F treated. (D) EndoH, endoglycosidase H treated. (E) to (H) are incubation with [¹²⁵I] α-fucosidase (2,00,000 cpm/ml), (E) incubation with untreated (normal), (F) with ALP treated, (G) with PNGase F treated and (H) with EndoH treated. The band at the top is due to the binding of radioiodinated enzyme to MPR 300 protein and the bottom is due to its binding to MPR 46 protein. The dephosphorylated and deglycosylated forms of the enzyme failed to bind the MPRs present on the membrane.
Figure 4B.4.3

$[^{125}\text{I}] \beta$-galactosidase

$[^{125}\text{I}] \alpha$-fucosidase

Figure 4B.4.3
Figure 4B.4.4: Internalization of $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase by $B_g$ cells. The cells were incubated with radioiodinated enzymes for 30 min at $28^\circ\text{C}$ with or without pre-incubation (30 min) of other ligands in serum free medium. (A) and (B) are the bar graphs plotted by taking % of internalized $[^{125}\text{I}]\beta$-galactosidase and $[^{125}\text{I}]\alpha$-fucosidase enzyme on Y-axis, respectively. C, in the figure represents control (incubation with radioiodinated enzyme alone). G6P, M6P, D-man, Im and PI represent prior incubation with glucose 6-phosphate, mannose 6-phosphate, D-mannose, Immune serum ($\textit{unio}$ MPR 300 antiserum) and preimmune serum, respectively. ALP, PNGase F and EndoH represent incubation with respective enzymatic treated radioiodinated enzyme. The bar graphs were plotted in Sigma plot 9.0 taking the average of triplicates. (C) At the end of incubation, the $B_g$ cell lysate was loaded on 10% SDS-PAGE, gel dried and developed by autoradiography. Top panel is due to $[^{125}\text{I}]\beta$-galactosidase and bottom panel is due to $[^{125}\text{I}]\alpha$-fucosidase. The bands developed only in control, G6P, D-man and PI incubated cells.
Figure 4B.4.4

A

% of β-galactosidase endocytosed

Ligand

G6P
M6P
D-man
Im
PI
PNGase F
EndoH

B

% of α-fucosidase endocytosed

Ligand

G6P
M6P
D-man
Im
ALP
PNGase F
EndoH

C

β-galactosidase

α-fucosidase

Control
G6-P
M6-P
D-mannose
Im
PI
ALP
PNGase F
EndoH

Figure 4B.4.4
Figure 4B.4.5: *In vitro* interaction between MPR 300 and radioiodinated enzyme; (top panel) immunoprecipitation of MPR 300 and $[^{125}I]$ β-galactosidase complex using *unio* MPR 300 antiserum and eluting the bound $[^{125}I]$ β-galactosidase from the complex. Lane1, elution with 5 mM glucose 6-phosphate (G-6-P), lane 2, elution with 5 mM mannose 6-phosphate (M-6-P) and lane 3, elution with 5 mM D-mannose. (bottom panel) immunoprecipitation of MPR 300 and $[^{125}I]$ α-fucosidase complex using *unio* MPR 300 antiserum and eluting the bound $[^{125}I]$ α-fucosidase from the complex. Lane1, elution with 5 mM glucose 6-phosphate (G-6-P), lane 2, elution with 5 mM mannose 6-phosphate (M-6-P) and lane 3, elution with 5 mM D-mannose.
Figure 4B.4.5

β-galactosidase

α-fucosidase

1 2 3

G-6-P  M-6-P  D-mannose
Figure 4B.4.6: Internalization of FITC-conjugated α-fucosidase by Bg cells; (A) incubation with FITC-conjugated α-fucosidase (green). (B) and (F) incubation with unio MPR 300 antiserum followed by Cy5 (red) conjugated secondary antibody. (E) Pre-incubation with 5 mM M6P before adding FITC-conjugated α-fucosidase (green). (C) merged image of (A) and (B), and extensive colocalization of α-fucosidase with MPR 300 can be seen. (G) merged image of (E) and (F). (D) and (H) are the corresponding transmission images. There is no fluorescence observed due to FITC in image (E), as the M6P has saturated the binding sites of MPR 300 present on cell surface. Bar in the top panel is 14.39 µm and the bottom panel is 22.3 µm.
Figure 4B.4.6
4B.5. DISCUSSION

The main objective of this investigation is to determine the binding kinetics of MPR 300 from \( Bg \) cells towards \( \beta \)-galactosidase and \( \alpha \)-fucosidase. Throughout our study we have used these two lysosomal enzymes which are from different sources, \( \beta \)-galactosidase was from bovine and \( \alpha \)-fucosidase from \( unio \). The \( \beta \)-galactosidase and \( \alpha \)-fucosidase were radioiodinated with \(^{125}\text{I}\) and used to study the saturation kinetics. The \( Bg \) cells were incubated with either of the radioiodinated enzyme in the concentrations ranging from 5-50 nM. The non-specific binding was determined using 2 \( \mu \)M non-radioiodinated enzyme for each of the concentration. The scatchard plot analysis gave \( K_d \) values of 22.52 nM and 8.090 nM for \( \beta \)-galactosidase and \( \alpha \)-fucosidase, respectively. The high affinity of \( \alpha \)-fucosidase (2.7 fold higher) over \( \beta \)-galactosidase can be attributed to the species specificity; the \( \alpha \)-fucosidase used was from the same species \( unio \).

Like many other glycoproteins, soluble lysosomal proteins are synthesized in the endoplasmic reticulum and are cotranslationally glycosylated by the transfer of high mannose oligosaccharides to specific asparagine residues (Kiely et al., 1976; Bergman et al., 1978; Rothman et al., 1978). As these proteins move through the secretory pathway, the lysosomal proteins are selectively recognized by a phosphotransferase that initiates a two-step reaction that results in the generation of the M6P modification on specific \( N \)-linked oligosaccharides (Lazzarino and Gabel, 1988; Gabel et al., 1989). These \( N \)-glycosylated proteins are sensitive for endo H and PNGase F enzyme cleavage (Trimble et al., 1978; Tarentino et al., 1985). We used these two glycosidases to investigate the importance of glycosylation in binding and endocytosis by MPR 300. In the ligand blotting experiment using untreated radioiodinated enzymes as probes showed binding to both the receptor proteins MPR 300 and 46 (Fig 4B.4.3A and E). Where as, the deglycosylated radioiodinated enzymes failed to bind either of the receptors (MPR 300 and 46) (Fig 4B.4.3B to D and F to H) indicating that the attached oligosaccharides were of highmannose or hybrid type and are essential for its interaction with MPRs. In internalization experiment also, the treated radioiodinated enzymes displayed a minimal uptake of 9-13% (Fig 4B.4.4). The importance of phosphate moiety was revealed by observing the inability of dephosphorylated radioiodinated enzymes to bind MPRs in ligand blotting and also being unable to get
internalized by the $Bg$ cells, as only a limited internalization in the range of 11-12% was seen.

In order to establish that the interaction between lysosomal enzyme and MPR 300 is M6P dependent we have used M6P, G6P and D-mannose in internalization experiments. Preincubation of $B g$ cells with M6P abolished the internalization by 93-95%, G6P and D-mannose showed only 12-18% inhibition. This signifies that M6P is clearly a crucial part of the enzyme recognition marker as originally inferred by Kaplan et al. (1977) and subsequently shown directly by several laboratories (Bach et al., 1979; Distler et al., 1979; Natowicz et al., 1979; von Figura and Klein, 1979). The ineffectiveness of G6P, the 2-epimer of M6P could be due to the loss of hydrogen bonds mediated by the axial 2-hydroxyl present in M6P or to the presence of an equatorial 2-hydroxyl that induces steric or other unfavorable factors. The specificity is further demonstrated in coimmunoprecipitation experiment, where in the bound radioiodinated enzyme was eluted specifically with 5 mM M6P from the MPR 300 immune complex pulled with protein A-agarose. The bound enzyme was not eluted with either G6P or D-mannose (5 mM each). The MPR 300 present on the cell surface is mainly involved in the endocytosis of exogenous ligands, to confirm that the internalization is primarily by the MPR 300 protein present on $B g$ cells, we have used unio MPR 300 antiserum in the internalization experiments with radioiodinated enzymes. The preimmune serum was used as a control to monitor the non-specific counts and it did not show any significant inhibition (10-11%), on the contrary the cells exposed to unio MPR 300 antiserum showed 93-94% inhibition in uptake. This tells us that the internalization was by MPR 300 and the antibodies have made the receptor protein inactive and subsequently unavailable for the radioiodinated enzymes.

The sub cellular localization of the endocytosed enzyme was followed by using FITC-conjugated $\alpha$-fucosidase and observing under confocal microscope. Here we have chosen $\alpha$-fucosidase as it was from the same species and moreover it exhibited higher affinity to MPR 300 in the binding experiments. The MPR 300 protein is usually detected in endosomes, in the TGN, and at the plasma membrane in varying proportions in different cell types (Willingham et al., 1983; Geuze et al., 1984; Griffiths et al., 1988; Press et al., 1998). In earlier studies, the MPR 300 has been used as a marker for late
endosomes (Goda and Pfeffer, 1988; Griffiths et al., 1988). So the same cells which are incubated with FITC-α-fucosidase were fixed permeabilised and immunostained with *unio* MPR 300 antiserum to detect MPR 300 protein. The merged image of FITC-α-fucosidase (green) and MPR 300 protein (Cy5, red) showed extensive colocalization in the endocytic compartments. The localization of the FITC-α-fucosidase in early and late endosomes is well in agreement with our understanding of its trafficking to lysosomes by the MPR 300. Prior incubation of the cells with 5 mM M6P did not result in internalization of FITC-α-fucosidase and no green fluorescence was seen in the cells.

In summary our results on $K_d$ determination for the MPR 300 from *Bg* cells is within the range of affinities previously published for vertebrate receptors (Tong et al., 1989; Tong and Kornfeld, 1989). The specificity of MPRs towards M6P in binding is also well in accordance with the previous reports (Ullrich et al., 1978, 1979). These studies have now laid the foundation to further study in detail additional features of the mollusc MPR proteins. In particular sequencing the MPR 300 protein, there by elucidating the structures of the M6P binding domains, analyzing the ability of the mollusc protein to bind human IGF-II (as the vertebrate receptors from fish to mammals bind human IGF-II) and to identify transport signals of the receptor protein, which is the future direction of work in the laboratory. These results would finally allow to draw an evolution tree for the MPR proteins.