Cloning and sequence determination of the

*Xiphophorus xiphidium* (A₂ cell) MPR 46
4.1. INTRODUCTION

In mammals, mannose 6-phosphate receptors (MPRs) play an important role in lysosome biogenesis by sorting newly synthesised acid hydrolases to lysosomes at the trans-Golgi network (TGN) (Dahms and Hancock, 2002; Ghosh et al., 2003). The occurrence of both MPRs (MPR 300 and MPR 46) in mammalian and non-mammalian vertebrates has been well characterized (Siva Kumar and Praveen kumar, 2010). The MPR 46 mediates intracellular sorting of lysosomal enzyme but does not endocytose extracellular ligands. It has been shown to mediate the secretion of lysosomal enzymes besides endogenous lysosomal enzyme sorting (Chao et al., 1990). In view of the distinct functions exhibited by the two receptor proteins, it is of interest to understand how these proteins were evolved.

The first report showing the existence of both MPRs in the early vertebrate fish came from our laboratory (Siva Kumar et al., 1999). Purified trout liver MPR 300 showed electrophoretic mobility similar to the purified goat liver receptor and a pH optimum of 6.5-7.0 for binding to phosphomannan. However the MPR 46 exhibited a lower molecular mass as compared to mammalian protein, possibly due to differential glycosylation. The molecular weight of the polypeptide in chicken and Xiphophorus was same (Udaya Lakshmi et al., 1999). Zebrafish MPR 46 has also been shown to be homologous to the other vertebrate receptors (Suresh et al., 2006). In order to gain more insights into the MPR targeting system in the fish, the present study was taken up with the following objectives, (i) to identify the lysosomal enzyme activities in the A2 cell extracts, (ii) check their cross-reactivity with the available lysosomal enzyme antibodies (iii) to prepare a full length cDNA for the fish MPR 46 receptor and to make a structural comparison of the sequences with other known receptors.
4.2. MATERIALS AND METHODS

4.2.1. Materials

Ham’s F-12 Nutrient medium (Gibco), foetal bovine serum (FBS) was purchased from PAN Biotech, India. 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 goat protein), arylsulfatase A antiserum (raised against human protein), affinity purified starfish cathepsin D IgG (raised against purified starfish cathepsin D protein); all antibodies used were polyclonal antibodies. Centrifugation steps in this study were done in Biofuge stratos centrifuge in 1.5 mL rotor unless otherwise mentioned.

4.2.2. Preparation of cell lysate and extraction of soluble proteins

*Xiphophorous xiphidium* (*A₂*) cells were cultured in complete medium (45 mL of Ham’s F-12 Nutrient medium, penicillin/Streptomycin (5μg/mL), 15% heat inactivated foetal 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 pellets were collected by centrifugation at 2991×g for 10 min. The pellet was suspended in 0.5 mL of 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 30 min. The supernatant obtained at this step is referred as acetate supernatant and was used for lysosomal enzyme assays. The lysosomal enzyme activities of α-fucosidase, α-galactosidase, β-hexosaminidase and α-mannosidase were assayed using p-nitrophenyl derivatives of the respective
substrates and arylsulfatase A was assayed using 4-Nitrocatechol sulfate dipotassium salt as described (section 3.2.2). The pellet was again resuspended with 0.5 mL of 50 mM imidazole buffer pH 7.0 containing 0.5% Triton X-100, 1mM PMSF, 5mM iodoacetic acid, 1mM EDTA to extract the membrane proteins as described above.

4.2.3. Immunoblotting

For immunological detection, 25 µg soluble protein was separated on a 10% SDS-PAGE and transferred onto a 0.45 mm pore diameter PVDF membrane (Immobilion-P, Millipore, Bedford, USA) as described (Towbin et al., 1979). The membrane was placed in blocking solution containing 5% non-fat milk powder in phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% Tween 20 (PBST) for 1.5 h. The blot was briefly washed with PBST and incubated overnight with the primary antibody in 1% milk with PBST. The following primary antibodies were used for the detection of the respective enzymes: Arylsulfatase A antiserum, dilution of 1:500; α-fucosidase antiserum, dilution of 1:300; β-hexosaminidase antiserum, dilution of 1:200; affinity purified starfish cathepsin D IgG, dilution of 1:500. The source of the antiserum used is described in section 4.2.1. The immunoreactive bands were visualized using a secondary antibody conjugated with HRP enzyme (Bangalore Genie) and detected by enhanced chemiluminescence using Super-Signals West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

4.2.4. Isolation of total RNA

Total RNA was isolated from the cold cell pellet using an acid guanidinium thiocyanate–phenol-chloroform mixture commercially available as TRIzol (Invitrogen). The quality of the RNA was confirmed by using a Bioanalyzer 2100 (Agilent Technologies) to ensure samples with intact 18S and 28S ribosomal RNA
peaks, and the amount was determined with a NanoDrop spectrophotometer (Thermo Scientific).

4.2.5. ReverseTranscritptase - Polymarase Chain Reaction

From the RNA obtained above cDNA was synthesized using 0.5 ng of oligo (dT) primer and 200 units of reverse transcriptase enzyme in a total volume of 20 µL (according to the manufacturer’s protocols; MBI Fermentas, India). The cDNA synthesized was used as template for amplification of the putative A₂ MPR 46 using the following primers.

 Sense primer: 5’-CGGAATTCATGACGGTGCAC-3’.
 Antisense primer 5’CCGCTCGAGTCACATCGGTAGCAA-3’. PCR was carried out in a total volume of 20 µL, 1µL (10 pM) each of primers, 2 µL of oligonucleotides, 4.5 µL of cDNA template, 10.5 µL nuclease free water and 1 µL of Taq DNA polymerase. After Taq DNA polymerase was activated by heating at 95°C for 5 min, the PCR reaction proceeded for 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 1 min at 72°C (extension).

4.2.6. Cloning and transformation of A₂ cell MPR 46

The PCR product was analyzed on 1% agarose gel electrophoresis. The single band obtained was excised, gel purified using Qiagen PCR Purification kit and subjected to TA cloning into pTZ57R vector (MBI Fermentas). The product was transformed into DH5α competent cells. The positive clones were selected by blue-white selection and the plasmid DNA was isolated by mini kit (Qiagen, India). The size of the insert was confirmed by restriction digestion of the plasmid DNA with EcoR1 and Xba1 enzymes (MBI Fermentas).
4.2.7. DNA sequencing

DNA sequencing was done at Biosereve sequencing Pvt.Ltd. Sequence comparisons were performed with the CLUSTAL W method available online.
4.3. RESULTS

4.3.1. Enzyme assays and immunoblotting of lysosomal enzymes

To detect the lysosomal enzyme activities in A2 cells, acetate supernatant from the cells was assayed using chromogenic substrates. Of the five lysosomal enzymes assayed viz., arylsulfatase A, \( \alpha \)-fucosidase, \( \alpha \)-galactosidase, \( \beta \)-hexosaminidase and \( \alpha \)-mannosidase, \( \beta \)-hexosaminidase showed very high enzyme activity for the same amount of protein taken (Fig 4.4.1). In order to identify the respective lysosomal enzymes, the soluble proteins were subjected to 10% SDS-PAGE and probed using the respective enzyme antiserum as described in materials and methods. Arylsulfatase A and cathepsin D showed a single band (Fig 4.4.2 A & D), \( \beta \)-hexosaminidase showed a band corresponding to 60 kDa and additional two bands above it which might be a precursor form of the enzyme (Fig 4.4.2B). \( \alpha \)-fucosidase showed a major band corresponding to 50 kDa. Two additional bands seen below the \( \alpha \)-fucosidase protein in Fig 4.4.2C which could represent a cross-reactive protein or a proteolytic product of the enzyme.

4.3.2. RT-PCR and Sequence Analysis

The quality of the RNA was confirmed by using a Bioanalyzer 2100 (Agilent Technologies) as shown in Fig 4.4.3. The amount was determined with a NanoDrop spectrophotometer (Thermo Scientific). Using the primers mentioned under methods a putative A2 MPR 46 gene was amplified (Fig 4.4.4A lane 2) which was then cloned into TA cloning vector. The presence of insert was confirmed by double digestion of the construct with EcoR1 and Xba1 (Fig 4.4.4B lane 3), lane 2 shows the undigested A2 MPR 46 gene into vector. Sequence was confirmed by automated DNA sequencing. Nucleotide and deduced amino acid sequence of the A2 fish CDMPR is presented in Fig 4.4.5. The deduced amino acid sequence of the proposed MPR 46
revealed that there is a 40% identity between the fish and mammalian CDMPRs (Human, bovine, goat and mouse). Interestingly, multiple sequence alignment of the fish CDMPR with other known mammalian CDMPR (Fig 4.4.6) further reveals that the amino acid residues, Q$^{92}$, H$^{131}$, R$^{137}$, E$^{159}$, R$^{161}$ corresponding to the human CDMPR, which has been implicated for direct involvement in M6P ligand binding is also conserved. The six cysteine residues involved in disulfide bridge formation in the extracytoplasmic region of the known vertebrate MPR 46 proteins are also conserved in the *Xiphophorus xiphidium* protein. In addition the potential glycosylation sites and transmembrane domain are also partially conserved.
Figure 4.4.1. Lysosomal enzyme activities from the A2 cell acetate supernatant using the respective chromogenic substrates: *Para-nitrophenyl phosphate (pNP)* derivatives. Aryl: Arylsulfatase A, Fuc: α-Fucosidase, Gal: α-Galactosidase, Hex: β-Hexosaminidase, Man: α-Mannosidase. Assays were done in triplicates in three individual experiments and the results obtained were plotted using Sigma plot 9.0 taking the averages and standard deviation for the error bars.
Figure 4.4.1

Activity IU/mg of protein

Aryl  Fuc  Gal  Hex  Man

0.0  0.5  1.0  1.5  2.0  2.5  3.0
Figure 4.4.2. Immunoblotting of lysosomal enzymes from A2 cells
Proteins were separated on 10% SDS-PAGE and transferred onto PVDF membrane. Antibody probing was done with the respective antibodies and detected by chemiluminiscence. (A) Immunoblot with arylsulfatase A antiserum, only one form of the enzyme is observed; (B) Immunoblot with β-hexosaminidase antiserum, additional bands might represent precursor form of the enzyme, (C) Immunoblot with α-fucosidase antiserum, (D) Immunoblot with Cathepsin D antibody, only one form is observed indicated by the arrow. (→) indicates the position of the enzyme.
Figure 4.4.2
Figure 4.4.3. Workflow of RNA isolation
RNA isolation using Trizol

RNA quality check using Bioanalyzer 2100

Figure 4.4.3
Figure 4.4.4. Molecular cloning of *Xiphophorus xiphidium* (A₂ cells) MPR 46. (A) PCR amplification of A₂ MPR 46 gene using RT product as the template. Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, mixed DNA ladder (100 to 10,000 bp), lane 2, amplified product. (B) Restriction digestion analysis of the positive clone, lane 1, mixed DNA ladder (100 to 10,000 bp), lane 2, undigested A₂ MPR 46 gene into pTZ57R vector, lane 3, double digested A₂ MPR 46 into pTZ57R vector (with EcoRI and XbaI). (→) fragment released from the vector after digestion with EcoRI and XbaI.
Figure 4.4.4
Figure 4.4.5. Nucleotide and deduced amino acid sequence of the *Xiphophorus xiphidium* (A₂ cells) MPR 46 protein.
cDNA sequence

AAAACCTGGCTAGCGTTTAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTTCCAGTGTGGTGGAATTCATGACGGTGCACGGTAGCAGGATGCCATCACATTGCATACCTCTGCTGTGCTGGTAGTCTTTATGGCCCTTGCTGCTGGTGTGGGGGC
TGAACCGCTGTGGAGAAGAGCTGATGTGGTTGGTGATGAGAGTACTGAGTCCCAAATGGAAAAAGCCCTGCTGAAGAAACTAGAACCCCTGAGCCAAATAAGGTTTAACACGACTGTGGAGATTGGCACAACTGGAAACTATGCCTACCATTTCAGGGATAGGCAGTCTGGAAAGACTACAGTGATAGGAAGAATCAATGAAACCCAGGTCTTCAATGGAAGTGACTGGATCATGCTGATTTATAAAGGAGGTGATTCATA
TGGTAGGCACTGCAGTGGTGAGAAAAGAAGAGCTGTGATAATGATTTCTTGCAAGCGAGAATGCACTGGACTGGATCATGCTGATTTATAAAGGAGGTGATTCATA

Deduced Amino acid sequence

KPGRLTAWYRARIHSSVVEFMVHGSMPHCHTSAVLVMALAGVGAEPLEKSCDVFDEQESTSQMEKALLKLEPLSLQIRFNTVEIGTTGNYAYHFRVCREVNSHLDFAGLVMQDRQSGKTTVIGRINETQVFNGSDWIMLIIKGDGYRHCSGKRAVIMISCKRGITASSFSIIEEREKQTVSTSLRWTAVWLVQX

Figure 4.4.5
Figure 4.4.6. Multiple sequence alignment of amino acids of MPR 46 protein from different species. (●) indicates the conserved residues essential for M6P-binding; (♦) indicates residues where mutation resulted in reduced binding; (▼) indicates a potential N-glycosylation site; ($) indicate the cysteines paired in disulfide bonds; the underlined sequence represents the transmembrane domain; (*) marks identical amino acid residues; (:) related amino acid residues; (·) predominantly the same amino acid residue. (BOS, bovine; GOT, Goat; HOM, Human; MUS, Mouse; XIP, Xiphophorus).

Zebrafish MPR 46 functions in lysosomal enzyme targeting. In killifish MPR 46 ligand binding region is conserved. In fugu fish the R$^{137}$ residue in the ligand binding region is replaced by Ser$^{137}$. 
CLUSTAL 2.1 multiple sequence alignment

Figure 4.4.6
4.5. DISCUSSION

In the present study, lysosomal enzyme activities have been identified in A₂ cells by enzyme assays and the same were quantified. Although different lysosomal enzyme activities could be identified in the soluble extracts, their relative concentrations seemed different. Due to the limited availability of antibodies in our laboratory, we concentrated only on four lysosomal enzymes viz., arylsulfatase A, α-fucosidase, β-hexosaminidase and cathepsin D. The authenticity of the isolated enzymes was confirmed by their specific immuno-reactivity using the available antibodies in the laboratory as described under methods. These initial results suggest there might be some antigenic similarities among the enzymes from different species. In the vertebrate species tested, it is already known that these enzymes are specifically transported to lysosomes by the two distinct but homologous receptors MPR 300 and 46 which have also been identified in the fish in our laboratory.

The presence of both the receptors with the biochemical properties similar to the mammalian proteins has been established by earlier work using *Xiphophorus xiphidium* A₂ cells (Siva Kumar et al., 1999). Subsequent studies on the partial sequence of the A₂ cell MPR 300 protein revealed high degree of structural homology of the A₂ cell protein to that of the other vertebrate MPR 300 proteins. The study also confirmed the presence of repetitive cassette structures in the extracytoplasmic domain and the 3rd domain containing the critical arginine residue for ligand binding (Udaya lakshmi et al., 2000). Preliminary study was carried out in our laboratory to obtain amino acid sequence for the MPR 46 protein from A₂ cells (Raju, 2004). To obtain more relevant information on the A₂ cell MPR 46 protein in particular its structural similarity to other vertebrate and fish receptor sequences known, in the
present study a cDNA clone was isolated for the A₂ cell MPR 46 protein and was characterized. This was essentially carried out to also compare the sequences of the A₂ cell MPR 46 protein to the other known vertebrate MPR 46 sequences.

From the available literature information and our present study on the A₂ cell receptor, it is logical to conclude that the early vertebrate fish has distinct lysosomal enzymes as well as their targeting receptors and the mechanism of binding the enzymes to receptors seems to be similar from fish to mammals as the functional domains of the receptors are highly conserved across the species. These results support that different fish species have homologous MPR 46 proteins which might have similar functions as the well studied Zebra fish protein. It has been seen in the preceding chapters, the lysosomal enzymes and their receptors seem to be conserved also among the invertebrates and our results so far conclusively establish their conserved nature among the starfish, the highly evolved invertebrate.