STUDIES ON MANNOSE 6-PHOSPHATE RECEPTORS FROM NON-MAMMALIAN VERTEBRATES AND INVERTEBRATES

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Dr.N.Siva Kumar** and partly in collaboration with **Prof.Dr.K.von Figura's** group in Goettingen, Germany supported by UGC-DAAD. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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CERTIFICATE

This is to certify that this thesis entitled "Studies on Mannose 6-phosphate Receptors from Non-mammalian Vertebrates and Invertebrates" submitted to the University of Hyderabad by Ms. Udaya Lakshmi Yerramalla for the degree of Doctor of Philosophy, is based on the studies carried out by her under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude and indebtedness to ***Dr.*** *N.* ***Siva Kumar*** *for his excellent supervision, ever willing encouragement, unending help and abiding interest throughout the tenure of my Ph.D work. I also express sincere thanks for encouragement to carry out part of my Ph.D work in the laboratory of* ***Prof. Dr. K. von. Figura*** *(Institut fur Biochemie Und Molekulare Zellbiologie, Heinerich-Duker weg 12D,* ***Gottingen-37075, Germany).***

I am thankful to Prof. K. Subba Rao, Head, Dept. of Biochemistry and Prof. R. P. Sharma Dean, School of life sciences, University of Hyderabad for providing necessary facilities to carry out my work.

I am thankful to ***Prof. Dr.K.von Figura*** *German Mentor for supporting my application for the award of UGC-DAAD (German academic exchange service) fellowship and extending all the lab facilities. His spontaneous suggestions at the critical times were of great help. I also thank him for re-inviting me for the successful completion of the work.*

I wish to express my deep sense of gratitude to ***Dr.Annette Hille-Rehfeld*** *for her excellent supervision, unending help and ever willing encouragement throughout my stay in Germany. She was always been very pushy to have number of fruitful discussions together with Prof Figura.*

I thank the faculty of school of life sciences, who taught me basics in biochemistry.

I wish to thank my dearest friends for sharing my feelings, Special thanks to my friends Vashisht, Radha, Jayaraju, Suresh and Mallikarjun for their timely help.

I thank Dr. Thomas Dierks, Dr. Bernhard Schmidt, Dr. Peter Schu, Dr. Christian korner, Dr. Paul Saftig (Gottingen, Germany) for their valuable suggestions.

I thank all my colleagues Jutta, Bettina, Steffani, Rixa, Jens, An/a, Martin, Claudia and others for giving a friendly atmosphere in the German laboratory.

Special thanks to my dearest friend and research colleague Liliana Schalamanova (Bulgaria) for controlling my moods and trying to keep me very happy throughout.

I thank my friends Dr.Shankar, Dr.Narendra kumar, Dr.Prasad, Chitkala, Priya, Hari Simha, Jyothi, Rastogi who made my stay in Germany a most memorable one.

I thank Nadine, Frau Sander. Katja, and Martina for their technical assistance.

My sincere thanks to the non-teaching staff at the University of Hyderabad and Gottingen, Germany for their assistance, whenever it was required.

I am extremely grateful to my parents, whose endeavours made me what 1 am. I am also thankful to my brothers and sisters for their love, affection encouragement throughout my academic life.

I once again thank my supervisors and others who had been a source of inspiration and help during my work.

I greatly acknowledge the financial support received from CSIR (INDIA), DAAD (Deutsche Akademische AustauschDienst, Germany and DAAD Office New Delhi) and the Deutsche Forschungsgemein Schaft (SFB 525 Germany).

-Udaya Lakshmi Yerramalla

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ABBREVIATIONS

APS	Ammoniumperoxodisulfate
ATP	Adenosine triphosphate
P-M.E	p-mercaptoethanol
bp	base pair
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cpm	Counts per minute
cDNA	Complementary DNA
CTP	Cytidine triphosphate
dATP	Deoxyadinosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxy guanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
DTT	Dithiothreitol
DVS	Divinyl sulfone
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
FKS	fetal calf serum
Glu 6-P	glucose 6-phosphate
HEPES	(N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid))
HPLC	High performance liquid chromatography
IGF-II	Insulin like growth factor-II
kb	Kilo basepair
kDa	Kilo Dalton
LB	Luria Bertani
Man6-p	Mannose 6-phosphate

min	Minute
µCi	micro Curie
ML 1J/ML III	Mucolipidosis
MOPS	Morpholino propane sulfonic acid
MPR	Mannose 6-phosphate receptor
tun	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PH	-log (H^+) concentration
Pen/Strep	Penicillin/ Streptomycin
pfu	Plaque forming unit
PMP	Pentamannosyl phosphate
I'M	Phosphomannan
pmol	Picomole
PPO	2,5 Diphenyloxazole
R_f	Relative front
rRNA	Ribosomal RNA
rpm	Rotations per minute
RT	Reverse Transcription
SDS	Sodium dodecyl sulfate
Taq	*Thermophilus aquaticus*
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylendiamine
TFA	Trifluroacetic acid
TGN	*trans*-Golgi network
T_M	Melting temperature
TPCK	N-tosyl-L-phenylalanylchloromethyl ketone
Tris	Tris-(Hydroxymethyl) aminoethane
UDP	Uridine diphosphate
UMP	Uridine monophosphate
1)V	Ultraviolet

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CHATER I

INTRODUCTION

Intracellular Protein Trafficking and Mannose 6-phosphate Receptor Mediated Lysosomal Enzyme Transport:

All cells constantly produce proteins of different destinations that must be transported from the site of synthesis in the cytoplasm, into or through various membrane structures. One of the fundamental questions in cell biology is how proteins made in one subcellular compartment specifically and efficiently reach their final destination. Studies of protein transport and localization have been carried out in organisms with diverse structures such as bacteria, yeast, plant and mammalian cells. The receptor mediated trafficking of acid hydrolases to lysosomes is the best understood pathway by which proteins are co-translated into the endoplasmic reticulum are segregated and delivered to their ultimate destinations (for review see von Figura and Hasilik., 1986; Pfeffer., 1988; Kornfeld and Mellman., 1989; Kornfeld., 1992; Hille - Rehfeld., 1995; Pohlmann., 1996).

Mannose **6-phosphate Receptors in the Intracellular Transport of Lysosomal Enzymes:**

In eukaryotic cells, the role of mannose 6-phosphate receptors **(MPRs)** in the specific sorting and targeting of lysosomal enzymes to lysosomes is well established. Lysosomes are acidic and high density intracellular organelle with unique membrane proteins. They are responsible for the degradation of both internalized and endogenous (endocytic and autophagic pathway) macromolecules into simpler substances. They are known to contain unique collection of acid hydrolases such as proteases, glycosidases, nucleases, phosphatases, and lipases. In multicellular organisms, the site at which these enzymes are synthesized and the mechanism by which these lysosomal enzymes are ferried to lysosomes is very well established.

Their specific sorting and transport to lysosomes involves three levels of recognition. The first step is the synthesis of lysosomal enzymes on the membrane bound ribosomes together with

other proteins (cell surface membrane proteins and secretory proteins) at the rough endoplasmic reticulum (RER), insertion into the lumen of RER by the cleavable signal peptide present on them. These proteins are co-translationally glycosylated by the transfer of a preformed high mannose oligosaccharide ((Glc)3, $(Man)_9$, (GlcNAc)2) from a dolichol intermediate onto the selected aspargine residues (Li *et al.*, 1978), followed by the trimming of glucose residues (Kornfeld *et al,* 1978). These newly synthesized glycoproteins in the endoplasmic reticulum with different destinations are sequestered into membrane bound vesicles and transported to the Golgi complex. As these newly synthesized glycoproteins migrate through the ER and Golgi apparatus, their oligosaccharide chains may be further processed in a differential manner (Tabas and Kornfeld., 1980).

The second level of recognition is the biosynthesis of the recognition marker, which occurs in the early region of the Golgi apparatus (Pohlmann *et al.*, 1982; Pelham 1988), where the acid hydrolases are recognized and acted upon by two distinct enzymes. The first enzyme N-acetylglucosaminyl-1-phosphotransferase, transfers N-acetylglucosaminyl-1-phosphate from UDP-N-acetylglucosamine to the 6-hydroxyl group of terminal mannose residues on the high mannose oligosaccharide chains of the acid hydro lases (first demonstrated by Reitman and Kornfeld 1981 in a microsomal fraction from rat liver). The second enzyme, N-acetyl glucosamine-1-phosphodiester α-N-acetylglucosaminidase, removes the covering N-acetylglucosamine residue which is in phosphodiester linkage to mannose residues on the oligosaccharide chains, thus resulting in the generation of mannose 6-phosphatemonoesters (Varki and Kornfeld., 1980; Waheed *et al,* 1981). The concerted action of these two enzymes produces the mannose 6-phosphate recognition marker, which converts the acid hydrolases to high affinity ligands for the Man-6-Phosphate receptor (Sly and Fischer 1982, von Figura and Hasilik 1986) **(Figure. 1).**

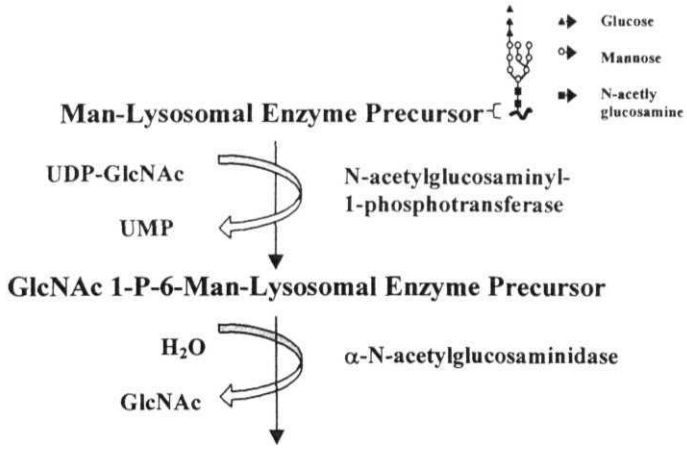


Figure 1: Two step biosynthetic process for the generation of terminal mannose 6-phosphate recognition marker in lysosomal enzymes

The third level of recognition involves segregation and binding of these high affinity ligands by specific membrane receptors in the Golgi apparatus. The MPR residing within the *trans*-Golgi network (TGN) is an integral membrane glycoprotein that binds the acid hydrolases through their Man 6-P recognition markers, after which they are concentrated and pinched off into clathrin coated vesicles (Brown and Farquhar 1984a). These vesicles then fuse with the prelysosomal compartment where the receptor and ligand complex dissociate by virtue of low pH, while lysosomal enzymes are somehow taken to mature lysosomes and the mannose 6-phosphate receptors recycle back to the TGN for another transport cycle (**Figure 2).**

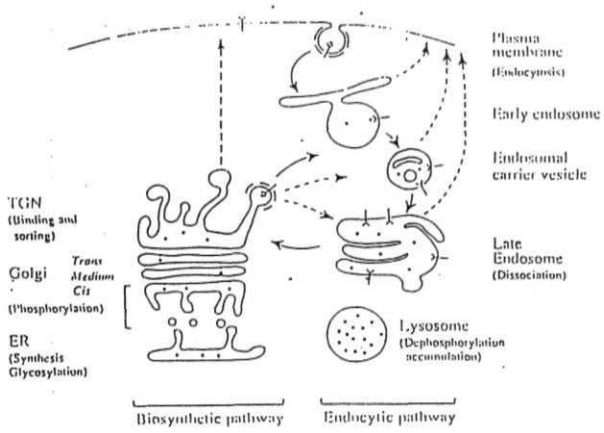


Figure 2: Model of mannose 6-phosphate receptor mediated lysosomal enzyme targeting (Hoflack and Lobel., 1993). After synthesis and acquisition of the M6P recognition marker, lysosomal enzymes (black dots) bind to MPRs (Y) in the TGN. The receptor-lysosomal enzyme complexes leave the TGN in clathrin coated vesicles which travel to and fuse with endosomes. After dissociation of the ligand and receptor due to acidic pH (most likely in late endosomes), the MPRs recycle back to the TGN while lysosomal enzymes are transported to lysosomes. Cell surface MPRs are involved in the internalization of mannose 6-phosphate containing ligands via clathrin-coated vesicles and fuse with endosomes, from where the lysosomal enzymes are transported to lysosome and the receptor recycles back either to the plasma membrane or TGN. The arrows show the pathway followed by the MPRs, dashed lines indicate transport steps which are uncertain.

Significance of the Mannose 6-phosphate Recognition Marker:

Hickman and Neufeld (1972) proposed the role of a common recognition factor acting in the segregation of lysosomal enzymes following their studies on fibroblasts from patients with I-cell disease, a condition in which the fibroblasts exhibited hyper secretion of lysosomal enzymes into the extracellular medium. Subsequent studies (Hickman *et al.*, 1974) showed that I-cell fibroblasts appeared to be capable of effectively endocytosing lysosomal enzymes secreted by

normal fibroblasts, in contrast, enzymes secreted by mutant fibroblasts were not susceptible to endocytosis by either mutant or normal cells. It was also reported that I-cell fibroblasts, unlike normal fibroblasts failed to incorporate ^{32}P into newly synthesized acid hydrolases (Hasilik *et al.,* 1981).

Later it was identified that the failure for the synthesis of M6-P markers results in the missorting of the lysosomal enzymes which is the basis for the rare disorder, I-cell disease (Hasilik *et al.*, 1981). Due to the defect of the phosphotransferase, the soluble hydrolases cannot be targeted to lysosomes in cells resulting in the accumulation of undigested substrates (inclusions). The analysis of I-cell disease greatly contributed to the discovery of the M6-P recognition marker dependent transport of lysosomal hydrolases and their cognate receptors.

In the cultured I-cell fibroblasts, a remarkable change in the distribution of mannose 6-phosphate receptors was found with an accumulation of coated vesicles bearing MPRs in Golgi region, but receptors were not detected in endosomes and lysosomes (Brown and Farquhar 1984b). Hieber *et al.,* (1976) found that mannose acted as a weak but stereospecific inhibitor for the endocytosis of bovine testicular β-galactosidase. Kaplan *et al.*,(1977) demonstrated, both yeast phosphomannans and mannose 6-phosphate as potent inhibitors for the endocytosis of the high uptake form of platelet β-glucoronidase.

Isolation and Characterization of Two Distinct Mannose 6-Phosphate Receptors:

Species from Yeast genus *Hansenula holstii* NRRL Y- 2448, was known to produce phosphomannans which are phosphorylated mannose polymers (Slodki.,1961). These phosphodiester containing phosphomannans were shown to have a mannose/ phosphorus molar ration of 5. The acid hydrolysis of *O*-phosphonomannan yielded two major components,

phosphomannan (PM) and pentamannosylphosphate (PMP) (Bretthauer *et al.*, 1973). PM and PMP were coupled to CNBr activated Sepharose and were used as affinity ligands for the purification of MPRs from various species (Stein *et al,* 1987a; Jeffrey *et al.*, 1975). Distler and Jourdian (1987) have prepared an affinity matrix by coupling PMP to co-amino ethyl substituted agarose and used for the purification of MPRs from bovine testes.

Extensive work carried out on these purified receptors clearly demonstrated that there exists two distinct phosphomannosyl receptors of different size and function. Both are type I transmembrane glycoproteins and are involved in the intracellular transport of lysosomal enzymes. A receptor with an apparent molecular mass of 275-300 kDa, has been purified from bovine liver using lysosomal enzyme β-galactosidase affinity matrix (Sahagian *et al.,* 1981), later it was also purified from human fibroblasts and swarm-rat chondrosarcoma cells, using yeast phosphomannan-Sepharose affinity column (Steiner and Rome., 1982). In addition Fischer *et al.,* (1982) purified bovine liver MPR by affinity chromatography, employing lysosomal enzyme secretions from *Dictyostelium discoideum.* This receptor binds mannose 6-phosphate containing ligands independent of divalent cations and hence designated as CI-MPR **(CI-MPR/ MPR 300)** and participates in transport of endogenous newly synthesized lysosomal enzymes **(Synthetic pathway)** and also in targeting the exogenous lysosomal enzymes through receptor mediated endocytosis to lysosomes **(Endocytic pathway).**

A second receptor has been purified by Hoflack and Kornfeld (1985a) from $P338D_1$ mouse macrophages (lacking MPR 300) and bovine liver. Later it was also purified from human liver (Stein *et al,* 1987a). It is a heterogeneous protein with an apparent molecular mass of 42-46 kDa and differs from the large receptor in that it requires divalent cations for optimal binding to its ligands *in vitro,* hence designated as CD-MPR (CD **MPR/ MPR** 46) (Hoflack and Kornfeld.,

1985a, 1985 b). It is immunologically distinct from the CI-MPR, but it has been suggested that both the receptors mediate the transport of lysosomal enzymes from Golgi to lysosomes. Separation of the two receptors was achieved by the selective binding of the large receptor to an affinity matrix containing methyl-6-phosphomannosyl residues present in lysosomal enzymes from *Dictyostelium discoideum.*

Role of pH in the Intracellular Transport of Lysosomal Enzymes:

Gonzalez-Noriega *et al.,* (1980) proposed that the acidification of transport vesicles is a crucial step for the release of bound hydrolases from MPR. A good evidence has been provided by the lysosomotropic amines, which raise intralysosomal pH from 4.5 to 6.0 resulting in the inhibition of uptake of exogenous acid hydrolases (Wiesmann *et al.,* 1975; Sando *et al.,* 1979; Ohkuma and Poole., 1978). In addition, it has been reported that the rate of lysosomal enzyme secretion by normal fibroblasts under the influence of these agents was greatly increased. However, the secreted enzymes were not defective in their recognition signals but it was proposed that, the rise in intralysosomal pH caused by amines might inhibit the release of hydrolases from the MPR. Blockage of the receptors with non-dissociating enzymes would prevent recycling of receptor, thus resulting in the secretion of subsequently synthesized lysosomal enzymes. Subsequent ligand binding studies with the purified MPR at varied pH were consistent with the proposal that pH is a critical parameter for MPR binding and transport of lysosomal enzymes to lysosomes (Sahagian *et al.,* 1981). From the above observations it was concluded that, binding of lysosomal enzymes occurs in the Golgi complex near to neutral pH and dissociation of the receptor and ligand occurs in the pre-lysosomal compartment by virtue of acidic pH. MPR 300 has a broad pH optimum of 6.0-7.5 for binding to its ligands and binds independently of divalent cations, whereas MPR 46 has a narrow pH optimum of 6.0-6.3 and the binding was enhanced in the presence of divalent cations *in vitro* (Tong and Kornfeld., 1989).

Intracellular Localization of MPRs:

Biochemical, morphological, cytochemical, immunocytochemical and subcellular fractionation techniques have been employed to study the intracellular localization of these receptors. These studies have shown that the receptor was found in the plasma membrane (Sahagian *et al* 1981), endosomes (Dickson *et al* 1983), Golgi membrane (Brown and Farquhar 1984a), and coated vesicles (Sahagian and Steer 1985), but are absent in mature lysosomes. However the relative amounts of both receptors in the different intracellular compartments as well as their distribution in the same animal and different animal species needs to be precisely resolved.

Relative Role of Two MPRs in Lysosomal Enzyme Sorting:

Several attempts have been made to define the relative roles of the two MPRs in the sorting of newly synthesized and endocytosed lysosomal enzymes. Immunological studies using anti-receptor antibodies for inhibiting ligand binding to receptor in various cell types such as cells expressing both the receptors and cells expressing only CD-MPR, indicated that both receptors participate in ligand binding but with different affinities (Gartung *et al.,* 1985; Stein *et al* ., 1987b). In cells expressing both receptors, blockade of MPR 300 by endocytosed antibodies against MPR 300 caused an increased missorting, whereas endocytosed antibodies against MPR 46 did not affect intracellular sorting. The lysosomal enzyme missorting due to antibody blockade of MPR 46 was evident only when MPR 300 had also been blocked by antibodies, or in cells which are deficient for MPR 300. This was further supported by cDNA (encoding MPR) transfection studies in CI-MPR deficient murine tumor cells expressing MPR 46, where the hyper secretion (60-75%) of newly synthesized lysosomal enzymes was completely corrected (reduced to 3%) by overexpression of CI-MPR and partially corrected (reduced to 30-35%) by overexpression of CD-MPR (Gabel *et al,* 1983; Kyle *et* ***al,*** 1988; Lobel *et al.,* 1989).

These studies concluded that CD-MPR is less efficient than CI-MPR in lysosomal enzyme sorting.

Mannose 6-Phosphate Receptors as Animal Lectins:

Since these two receptors share no obvious sequence similarity with other lectins (Carbohydrate binding proteins), it has been proposed that the two MPRs contain a distinct class of sugar binding domains, hence they were classified as **P-type lectins** (Drickamer and Taylor., 1993).

Primary Structure of MPRs:

Both mannose 6-phosphate receptors have been cloned and sequenced at the cDNA level from various species **(Table 1).** Both receptors are integral membrane proteins with three distinct domains, the extracytoplsmic domain, the transmembrane domain and the cytoplasmic domain **(Figure** 3; for review see Hille-Rehfeld., 1995; Pohlmann 1996).

***MPR** 46:*

Hoflack and Kornfeld in 1985 first discovered a cation dependent mannose 6-phosphate receptor with an apparent molecular mass of 46 kDa. The cDNA for the MPR 46 has been cloned and sequenced from several species such as human (Pohlmann *et al,* 1987), bovine (Dahms *et al,* 1987) and mouse (Koster *et al,* 1991; **Ma** *et al* ., 1991; Ludwig *et al,* 1992) and partially sequenced from chicken (Matzner *et* ***al,*** 1996). The mRNA of the human MPR 46 codes for a protein of 277 amino acids consisting of four structural domains viz., a N-terminal signal sequence of 20 or 26 amino acids, extracytoplasmic domain of 164-170 amino acids exposed at the plasma membrane or oriented to vesicle lumen, a single transmembrane domain of 20 amino acids followed by 67 amino acids of cytoplasmic domain. The short extracytoplasmic domain

was shown to exhibit 14-37 % homology to individual repeats of MPR 300 (Dahms *et al,* 1987, Lobel *et al.,* 1988). In contrast to this homology, there are no sequence similarities among the signal sequences, transmembrane regions and the cytoplasmic domains of the two receptors. MPR 46 contains 5 potential N-glycosylation sites, four of which are used (Wendland *et al.,* 1991). The carbohydrate portion contributes to 40% of the apparent molecular mass of the MPR 46. The position of cysteine residues which are most likely involved in disulfide bond formation are well conserved within the extracytoplasmic domain of MPR 46 (Lobel *et al.,* 1988). MPR 46 is a highly conserved protein with 93-95% overall homology from mouse to man and with completely identical amino acid sequence within the cytoplasmic domain of these species. The cytoplasmic domain contains a single casein kinase-II phosphorylation site. The gene for the human MPR 46 has been localized to chromosome 12 (Pohlmann *et al.,* 1987). The gene spans about 12 kb and consists of 7 exons (Klier *et al.,* 1991).

MPR 300:

The large cation independent mannose 6-phosphate receptor with an apparent molecular mass of 300 kDa was first isolated by Sahagian *et al*., (1981). The cDNA of MPR 300 has been cloned from several species such as man (Morgan *et al.,* 1987; Oshima *et al.,* 1988) bovine (Lobel *et al.,* 1987, 1988), murine (Mc Donald *et al,* 1988; Szebenyi and Rotwein 1994) and chicken (Zhou *et al*., 1995). The cDNA for the human MPR encodes 2491 amino acids, consisting of four structural domains viz., a signal sequence of 40 amino acids, a luminal domain of 2264 amino acids, a single transmembrane domain of 23 residues and a cytoplasmic domain of 164 amino acids. The extracytoplasmic domain consists of 15 repetitive units, each consisting of 147 amino acids in length with 14-28 % sequence homology with each other. It was investigated that repeat 13 from the amino terminus contains a 43 amino acid residue insertion

that is similar to the sequences found in fibronectin (Hynes., 1985). This segment forms a part of the collagen binding domain in fibronectin, but its function in the MPR 300 is unknown.

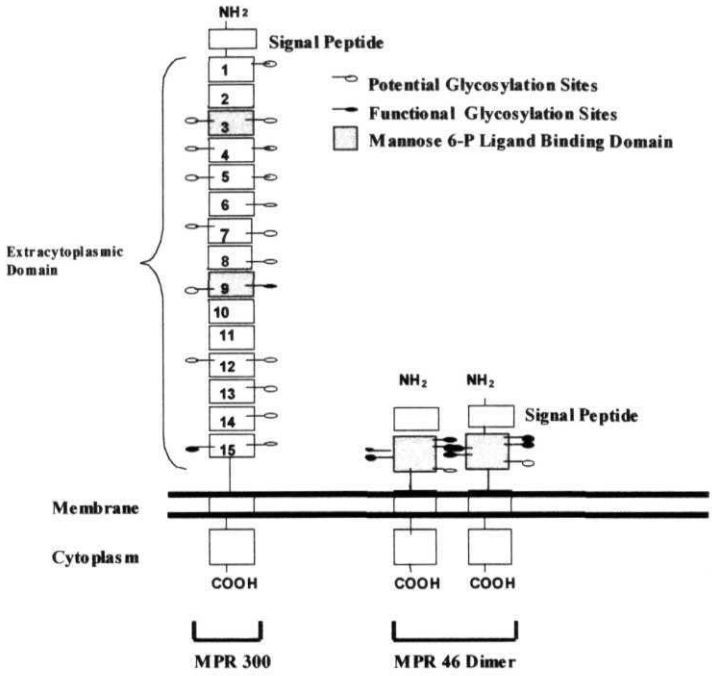


Figure 3: Schematic Representation of the Primary Structure of Mannose 6-phosphate Receptors. The repetitive units in the extracytoplasmic domain of MPR 300 are numbered from 1-15. The *in vivo* functional existence of monomeric and dimeric forms of MPR 300 and MPR 46 respectively are represented (according to Dahms *et al.,* 1989).

The position of the cysteine residues are highly conserved among the repeats. MPR 300 contains 19 potential glycosylation sites and only two of them are used. For MPR 300, the overall homology from mouse to man was found to be 82%. Although the cytoplasmic domains show considerable variation (80% homology from mouse to man), they retained functionally important stretches of highly conserved amino acids that are involved in endocytotic sorting, internalization, G-protein binding and casein kinase-II phosphorylation (Mac Donald *et al.,*

1988). The gene for MPR 300 has been localized to chromosome 6 in man and chromosome 17 in mouse (Laureys *et al.*, 1988). The gene spans about 130 kb in mouse (Stoger *et al.*, 1993) and consists of 48 exons (Szebenyi and Rotwein., 1994).

Post Translational Modifications of MPRs:

MPRs are known to undergo various post-translational modifications, some of them having functional significance. During biosynthesis of MPRs, N-glycosylation and formation of intra subunit disulfide bonds occur early after translocation into the endoplasmic reticulum (for review see Hille-Rehfeld 1995; Sahagian and Neufeld., 1983). It was reported that, among the four N-glycosylation sites of bovine MPR 46, the one at position Asn-87 was most important to facilitate folding and to partly stabilize the ligand-binding conformation (Zhang and Dahms., 1993). The exact role of carbohydrate moiety on MPR 300 for binding to mannose 6-phosphate containing ligands has not been investigated.

The presence of intra subunit disulfide bonds was found to be crucial for the formation of proper ligand-binding conformation in MPR 46 (Hille *et al* ., 1989). Replacing any of the six cysteine residues with glycine residues resulted in retention and degradation of MPR 46 within the endoplasmic reticulum (Wendland *et al.*, 1991). On the other hand, the highly conserved pattern of cysteine residues (6 to 8 per each domain) within each domain of MPR 300, suggests a similar importance of disulfide bond formation for this receptor (Lobel *et al*, 1988). Phosphorylation of serine residues in the cytoplasmic domain has been observed in both mannose 6-phosphate receptors (MPR 300 from human, bovine and hamster cells and also in human MPR 46) (Hemer *et al.*, 1993., Meresse *et al.*, 1990). Additional threonine phosphorylation was found in rat MPR 300 (Corvera *et al.*, 1988). The physiological

	MPR 46	MPR 300
Primary Structure		
cDNA Cloning	1. human (Pohlmann *et al.*, 1987) 2. bovine (Dahms *et al.*, 1987) 3. murine (Koester *et al.*, 1991., Ludwig *et al.*, 1992; Ma *et al.*, 1992) 4. chicken (Matzner *et al.*, 1996)	1. human (Morgan *et al.*, 1987; Oshima *et al.*, 1988) 2. bovine (Lobel *et al.*, 1987; Lobel *et al.*, 1988) 3. rat (Mac Donald *et* a/., 1988) mouse (Szebenyi and Rotwein. 4. 1994) chicken (Zhou *et al.*, 1995) 5.
Deduced amino acid sequence		
(Human MPR 46) signal sequence cxtracytoplasmic domain transmembranc domain cytoplasmic domain Internal repeats within cxtracytoplasmic domain	277 amino acids in total 20-26 164-170 20 67 1	2491 amino acids in total 40 2264 23 164 15 (~ 147 amino acids each) Fibronectin binding site in collagen showing -homology in repeat 13 M6P-binding site in repeat 3 and 9 IGF-II binding site in repeat 11
M_r of polypeptide Apparent M_r	30 kDa 43-46 kDa	270 kDa 275-300 kDa
Post-translational Modifications		
N-glycosylation sites Potential sites Glycosylated sites	5 2 high mannose 2 complex	19
Disulfide bonds Oligomerization Phosphorylation	3 pairs dimers, tetramers ser 56 (CK II)	3-4 pairs per repeat monomer, oligomer ? ser 82 (CK II) ser 157 (CK II)
Palmitoylation	not known	yes
Genomic Structure		
Location on chromosome	6 (murine)	6 (human) 17 (murine)
Gene length	12 kb	130 kb
Exon structure	7 exons in total	48 exons in total

Table 1: Protein Structure and Genomic Organization of Mannose 6-phosphate Receptors

(Hille-Rehfeld., 1995)

relevance of serine phosphorylation for sorting of mannose 6-phosphate receptors into clathrin coated pits at the TGN and/ or plasma membrane remained a matter of debate. Posttranslational modification by palmitoyl groups has been described for MPR 300 (Westcott and Rome, 1988). The structural features of both receptors are summarized in **Table 1.**

Oligomeric Structure of MPRs:

The oligomeric structure of the CD-MPR has been analyzed in several laboratories (Li *et al.*, 1990; Stein ***et*** *al.,* 1987; Dahms and Kornfeld., 1989; Waheed and Figura., 1990; Waheed ***et*** *al.,* 1990; Wendland *et al.,* 1989). These reports indicate that detergent-solubilized receptor can exist as a monomer, or non-covalently linked dimer or tetramer depending on the experimental conditions. The formation of dimeric and tetrameric forms is favored by low temperature ($<16^0$C), neutral pH, presence of mannose 6-phosphate and high protein concentration, whereas monomer formation is favored under the opposite conditions (Waheed *et al.*, 1990). Human monomeric CD-MPR did not bind to PM-Sepharose affinity column (Waheed *et al.,* 1990). However, the monomeric form of bovine receptor did bind to PMP-affinity column in the presence of divalent cation (Mn^{2+}) (Li *et al.,* 1990). These discrepancies may reflect species variation or possibly differences in affinity column used to assess the receptor binding ability.

It was observed that the truncated MPRs containing only the extracytoplasmic domain retained the ligand binding affinity, confirming that the transmembrane and cytoplasmic domains are not required for the formation of ligand binding conformation (Dahms and Kornfeld., 1989; Wendland *et al.,* 1989; Causin *et al.*, 1988). Chemical cross-linking experiments have revealed that the CD-MPR exists as a dimer in the membrane bound form. However Waheed *et al.*, (1990) observed monomeric, dimeric and tetrameric forms of the receptor in baby hamster kidney cells over expressing the CD-MPR. From these observations it was speculated that

changes in the quaternary structure of the receptor during recycling might influence the biological activity of this molecule. The three dimensional structure analysis also revealed that, the extracytoplasmic domain of the bovine CD-MPR crystallizes as a dimer, and each monomer folds into a nine-stranded flattened (3 barrel, which bears a striking resemblance to avidin (David *et al.,* 1998). It is consistent that Cl-MPR exists as a monomer in the membrane (Perdue *et al.,* 1983), but the chemical cross-linking experiments indicated that it may be an oligomer (Stein *et al,* 1987a).

Ligand **Binding Properties of MPRs:**

Equilibrium dialysis experiments indicated that the CD-MPR binds one mole of the monovalent ligand mannose 6-phosphate (M6-P) and 0.5 mole of a diphosphorylated high-mannose oligosaccharide per monomeric subunit. Consequently each dimer would have two M6-P binding sites, both of which can be occupied by a single oligosaccharide containing two M6-P residues. Studies showed that a complete loss of binding activity occurred following the reduction of the CD-MPR with dithiothreitol (Li *et al.,* 1990) or by the replacement of each of the six cysteine residues with gylcine (Wendland *et al.,* 1991) indicating importance of disulfide bonds in carbohydrate recognition. A three dimensional structure of a glycosylation-deficient, yet fully functional form of the extracytoplasmic domain of the bovine CD-MPR complexed with mannose 6-phosphate was provided by David *et al.* (1998). Analysis of the structure confirmed that the disulfide bond formation is important in the stabilization and /or orientation of the loop involved in M6-P binding. It was also reported that His-105 in CD-MPR is the most likely candidate to confer the pH dependence that is observed in phosphomannosyl binding. In addition, Asp-103 which coordinates the divalent cation may explain why CD-MPR exhibits enhanced binding in the presence of divalent cations (David *et al.,* 1998).

The CI-MPR on the other hand, binds two moles of mannose 6-phosphate or one mole of diphosphorylated oligosaccharide per monomer (Tong and Kornfeld.1989; Distler *et al.*, 1991; Tong *et al.*, 1989), Suggesting that only 2 of the 15 repeating units of the receptor may be involved in the M6-P ligand binding. Later it was investigated that, two independent M6-P ligand-binding sites have been shown to be localized on repeat 3 and 9 of bovine MPR 300. With chemical modification and site directed mutagenesis, it was concluded that, positively charged arginine residues in both the repeats were found to be critical for ligand binding activity (R435 in repeat 3 and R1334 in repeat 9; Dahms *et al.,* 1993). More recent studies carried out with mutant bovine CI-MPR containing substitutions for arginine residues at amino acid position 435/ or 1334, in comparison to the wild type receptor for their ability to sort lysosomal enzymes to lysosomes revealed that, MPR containing a single functional mannose 6-phosphate binding site are ~ 50% less efficient than the wild type. In addition to this, it was also observed that the two mannose 6-phosphate binding sites are not functionally equivalent in their ligand binding affinities (Patricia *et al.,* 1998). Site directed mutagenesis of each of the five histidine and the eight arginine residues of mature human CD-MPR revealed that His 131 and Arg 137 are critical for ligand binding (Wendland *et al.*, 1991). Ligand binding affinities of both receptors to various mannose 6-phosphate containing ligands are shown in the Table 2,

Ligand	**MPR 300**	**MPR 46**
M6-P	7-8 x 10^{-6} M	7-8 x 10^{-6} M
PMP	6×10^{-6} M	6×10^{-6} M
High mannose oligosaccharides with two phosphomonoesters or lysosomal enzymes	2 x 10^{9} M	2 x 10^{-7}M
Methyl 6-phosphomannosyl diesters (from *Dictyostelium discoideum*)	Shows a weak interaction	No binding

Table 2: Affinity constants of MPRs to various M6-P containing ligands

Multifunctional Ligand Binding Properties of MPR 300:

The mammalian MPR 300 (human, bovine and mouse) was found to be a multifunctional protein with a high affinity binding site for non-glycosylated insulin-like growth factor-II (IGF-II) hence designated as MPR 300/ IGF-II receptor (Tong *et al.,* 1988; Roth *et al,* 1987; Waheed *et al.,* 1988; Kiess *et al,* 1988). In contrast, the chicken and frog MPR 300 lack the high affinity IGF-II binding site (Canfield and Kornfeld., 1989; Clairmont and Czech., 1989). CD-MPR has also been shown to lack IGF-II binding affinity. The stoichiometry of IGF-II binding to the bovine receptor has been determined to be one mole of ligand bound per polypeptide with an affinity of 2×10^{-8} M (Tong *et al.,* 1988). The IGF-II binding site is localized on repeat 11 and the binding affinity was enhanced by repeat 13 (Schmidt *et al.,* 1995; Devi *et al.,* 1998).

The receptor located at the plasmamembrane, endocytoses secreted lysosomal enzymes, mediates internalization of growth factors such as IGF-II (Kiess *et al.,* 1988) and proliferin (Lee and Nathans., 1988) leading to their subsequent degradation intracellularly. It also potentiates the activation of the precursor form of TGF-P (latent TGF-p) into biologically active TGF-P (Dennis and Rifkin., 1991).

MPR 300/ IGF-II receptor is considered to be a tumor suppressor because of its ability to activate TGF-p (a potent growth inhibitor). It promotes degradation of the growth factor IGF-II and regulates localization of the lysosomal enzymes implicated in extracellular matrix degradation. Recent findings have shown that loss of MPR 300/ IGF-II receptor is an early event in the etiology of cancer (Hankins *et al.,* 1996). In the M6-P/IGF-II receptor mutant tumors, activation of TGF-p did not occur and was accompanied by excessive IGF-II expression (Wang *et al.*, 1997).

Binding and endocytosis of IGF-II by MPR 300 contributes to the regulation of embryonic development in mice. Other ligands binding to MPR 300 are retinoic acid (Kang *et al*., 1997) and urokinase receptor (Nykjaer *e t al.,* 1998), but the physiological relevance of their binding is not known yet. Recently it was also reported that a non-lysosomal enzyme, human DNase I an endonuclease involved in the hydrolysis of DNA into oligonucleotides under alkaline conditions, was also found to contain mannose 6-phosphate and binds to Cl-MPR (Cacia *et al*., 1998). Blanchard *et al* (1999) have reported another non-lysosomal highly glycosylated leukemia inhibitory factor (LIF) belonging to cytokine family to contain mannose 6-phosphate residues and this factor binds to MPR 300, resulting in the internalization and degradation of LIF. The binding of LIF to MPR 300 did not lead to signal transduction. From these observations it is quite evident that MPR 300/ IGF-II receptor plays an important role in regulating the amounts and biological activities of several non-lysosomal proteins ***in*** *vivo.*

Cytoplasmic Sorting and Internalization Motifs in MPRs:

Mannose 6-phosphate receptors mediate specific sorting and targeting of newly synthesized lysosomal enzymes to lysosomes. The mannsoe 6-phosphate receptor binding of lysosomal enzymes is followed by the sorting of the complexes into AP-1 (Golgi restricted) rich, clathrin coated vesicles at the TGN, fusion of these vesicles with endosomes, pH induced dissociation of receptors and ligands and sorting of these receptors into vesicles trafficking back to the Golgi. The receptors recycling between the cell surface and endosomes (endocytic pathway) are in equilibrium with those recycling between the TGN and endosomes (synthetic pathway) (Dahms *et al.,* 1989). Di-leucine and tyrosine based sorting motifs have been identified interacting with the assembly proteins AP-1 and AP-2 to promote the formation of TGN and plasma membrane derived vesicles respectively (Honing *et al.,* 1997; Denzer *et al.,* 1997).

Work from a number of laboratories suggests that, the complex recycling pathways of MPRs require several sorting signals which have been localized within the cytoplasmic domain (for review see Keen., 1990; Pearse and Robinson., 1990). Deletion of 67 residue cytoplasmic tail of MPR 46 (Figure 4) resulted in the accumulation of the truncated receptor at the cell surface (Peters *et al,* 1990). Internalization of the CI-MPR is directed by the sequence YKYSKV (Lobel *et al,* 1989; Canfield *et al,* 1991; Jadot *et al,* 1992); the CD-MPR uses a phenylalanine-containing sequence (FPHLAF) as well as a YRGV sequence (Johnson *et al,* 1990). These signals possibly interact directly with the plasma membrane-specific, AP-2 clathrin adapter complex (Glickman *et al,* 1989; Sosa *et al,* 1993).

In CI-MPR, a C-terminal di-leucine motif (LLHV) and the tyrosine-based endocytosis signal (YKYSKV) are important for efficient lysosomal enzyme delivery (Johnson and Kornfeld., 1992a) (Figure 4). The CD-MPR also contains a di-leucine signal (HLLPM) that is important for enzyme sorting (Johnson and Kornfeld., 1992a); thus this sequence may represent the binding site for the Golgi-specific, AP-1 clathrin adapter complex (Le Borgne *et al,* 1993). Although, significant progress has been made in identifying signals important for clathrin coated vesicular transport either from TGN to endosomes or from plasma membrane to endosomes. much less is known about how both MPRs are selected for export from endosomes to TGN or plasma membrane. More recently, a cytosolic 47 kDa hydrohpilic protein (TIP 47-tail interacting protein) was identified, which selectively binds to the cytoplasmic domains of both MPR 300 and MPR 46 and is required for transport of vesicles from endosomes to TGN or plasma membrane (Diaz and Pfeffer., 1998).

QRLVVGAKGM[10] EQFPHLAFWQ[20] DLGNLVADGC[30] DFVCRSKPRN[40] VPAAYRGVGD[50] DQLGEESEER[60] DDHLLPM A

KKERR[5] EM[7]-V[22]SYKYSKVNKEE[34]-D[153] DSSDEDLLHV[163] **B**

Figure 4: The cytoplasmic tails of human MPR 46 (A) and bovine MPR 300 (B). The sorting and internalization signals associated with sequence determinants are underlined

Mannose 6-phosphate Independent Pathway:

Neufeld and McKusick (1983) have identified mannose 6-phosphate independent pathway in various tissues from I-cell patients deficient in N-acetyl glucosamine phosphotransferase, where the activities of some lysosomal enzymes (β-glucocerebrosidase, and cathepsin D) must use mannose 6-phosphate independent mechanism for their transport. This observation was also confirmed by the studies on the targeting of lysosomal membrane glycoproteins (Lamp-1 and Lamp-2) and also lysosomal acid phosphatase which are devoid of mannose 6-phosphate residues (Lippincott-Schwarz and Fambrough., 1986; Waheed *et al*., 1988) and are transported to lysosomes independent of mannose 6-phosphate recognition system. This was speculated to be recognized by sorting signals within their cytoplasmic domains (Guarnieri *et al.*, 1993; Honing and Hunziker., 1995). Therefore, it is more convincing that mannose 6-phosphate independent transport does exist and contributes to the targeting of a few lysosomal enzymes and lysosomal membrane glycoproteins in normal tissues.

MPRs in Evolution:

In the evolution constituting large spectrum of animals showing great heterogeneity in their morphological and physiological characteristics **(Table 3),** only a few species have been the subject of investigation. No simple evolutionary picture can be expected to emerge from

comparison of non-mammalian vertebrates and invertebrates with highly evolved mammals. However, recognition of elements in particular MPRs of the mammalian pattern, in non-mammalian species would provide a picture of possible conservation of fundamental mechanisms for specific sorting and targeting of lysosomal enzymes to lysosomes in the vertebrate and invertebrate lineage (Figure 5).

So far, two distinct mannose 6-phosphate receptors (MPR 300 and MPR 46) have been identified and well characterized in mammals (for review see Hille-Rehfeld., 1995). The occurrence of both MPRs among the non-mammalian vertebrates such as aves (chicken), reptiles (garden lizard), amphibia *{Xenopus)* has been established by biochemical and immunological methods (Matzner *et al.,* 1996; Siva kumar *et al.,* 1997).

So far, the machinery used for the lysosomal enzyme sorting and targeting has been poorly studied in non-mammalian vertebrates and invertebrates. Lysosomal enzymes of *Dictyostelium discoideum* were shown to contain methylated mannose 6-phosphate residue, but receptors involved in mannose 6-phosphate specific sorting in *Dictyostelium* have not yet been characterized (Mehta *et al.,* 1996). Sorting of lysosomal enzymes (lacking mannose 6-phosphate) in the unicellular trypanosomes is thought to occur independent of mannose 6-phosphate (Oeltmann *et al.*, 1994; Heute-Perez *et al.,* 1999). Mannose 6-phosphate independent trafficking of the lysosomal proteases cathepsin D and L in mammalian cells has also been detected (McIntyre and Erickson., 1993).

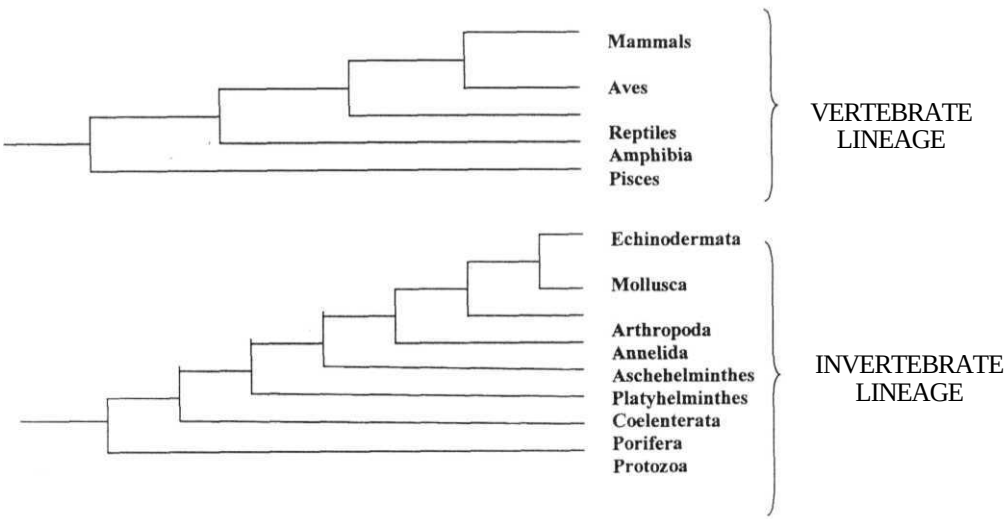


Figure 5: Vertebrate and Invertebrate Lineage with Phyla represented in the ascending order

Group	Cell Structure	Properties	Constituent group
Eucaryotes	Eucaryotic	Multicellular; Extensive differentiation of cells and tissues	Animals: vertebrates and invertebrates Plants: seed plants, ferns and mosses
		Unicellular, Coenocytic or mycelial; Little or no tissue differentiation	Protists (algae, fungi, protozoa)
Eubacteria	Procaryotic	Cell chemistry similar to eucaryotes	Most bacteria
Archaebacteria	Procaryotic	Distinctive cell chemistry	Methanogens, halophiles, thermophiles

Table 3: Primary Sub Divisions of Cellular Organisms that are now Recognized

On the other hand, the yeast *Saccharomyces cerevisiae* (primitive eucaryote) is known to contain large vacuoles which are functionally similar to the mammalian lysosomes in many ways. Two enzymes from *S.cerevisiae* have been reported to contain peripheral mannosyl phosphate and mannobiosyl phosphate in diester linkage to high mannose type oligosaccharides. The vacuolar enzyme carboxypeptidase-Y (CY) containing 4 Asn linked high mannose type oligosaccharides was found to be segregated into the vacuoles, independent of mannose 6-phosphate pathway, and extracellular form of the invertase enzyme also exhibited similar properties (Trimble and Maley., 1977).

OBJECTIVE OF THE PRESENT INVESTIGATION

It is well established that two distinct mannose 6-phosphate receptors localized in *trans*.Golgi.network and plasma membrane, play an important role in specific sorting and targeting of mannose 6-phosphate containing ligands (lysosomal enzymes) to lyosomes. Both the receptors (MPR 300 and MPR 46) have been well characterized from mammals (human, bovine, rat and mouse) and aves (chicken). Preliminary studies carried out by Siva Kumar *et al.*, (1997) provided the first biochemical evidence for the presence of both MPRs in reptiles (garden lizard) and amphibia (*Xenopus*).

Based on the primary structure of MPR proteins, it was predicted that, among the two receptors, MPR 46 is possibly the most ancient one with a short N-terminal domain. MPR 300 has been shown to have a long stretch of N-terminal domain consisting of 15 repetitive sequence domains, which could have been originated by gene duplication of MPR 46, as each one of the fifteen repeat units was showing sequence homology to the N-terminal domain of MPR 46.

Work from various groups established that mammalian MPR 300 is a multifunctional protein playing a crucial role in targeting of newly synthesized lysosomal enzymes to lysosomes and also has a high affinity binding site for insulin like growth factor-II. More recent reports indicate that several other non-lysosomal proteins such as proliferin, latent transforming growth factor, urokinase receptor, retinoic acid, human DNase 1 and Leukemia inhibitory factor also bind to mammalian MPR 300. The physiological significance of these multifunctional properties of MPR 300 is the subject of limited investigation although it was proposed that,

MPR 300 might be playing an important role in regulating the amounts of these non-lysosomal proteins *in vivo*.

The above characteristic features so far studied for mammalian MPR proteins prompted us to investigate in detail on the evolution of the MPR proteins using the following criteria. (i) To purify the MPR proteins from non-mammalian vertebrates such as chicken and fish, and invertebrates such as *Unio* employing the PM gel developed by us. (ii) At what stage during evolution both MPRs started appearing and among the two receptors which one of them is the most ancient one ? (iii) If they are present in other animal species do they share structural and functional properties similar to that of mammalian counterparts, if so to what extent?

With an effort to contribute to the above questions, the aim of the present study focussed on the following (1) To optimize the phosphomannan binding abilities of chicken MPR 46 (from chicken embryonic fibroblasts) on the affinity matrix developed (PM-gel), (2) To isolate mannose 6-phosphate receptor homologues from non-mammalian vertebrate (fish) and invertebrate (*Unio*), based on their phosphomannan binding abilities and specific elution with 5 mM mannose 6-phosphate (3) Isolation of fish MPR 46 from embryonic fish cells, employing similar conditions that were used for binding chicken MPR 46 to PM gel (4) RT-PCR amplification of fish cDNA fragments using MPR specific degenerate primers corresponding to the highly conserved regions of known mammalian and avian (chicken) MPR sequences (5) Retrieval of fish MPR specific cDNA sequences by library screening with fish derived MPR specific cDNA probes.

CHASTER II

BIOCHEMICAL CHARACTERIZATION OF NON-MAMMALIAN VERTEBRATE/ INVERTERBRATE 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	Pharmacia,Uppsala, Sweden
SMART-System	
Gels for SMART	
Gel filtration column-fast desalting PC 3.2/10 (3.2 x 100mm)	Pharmacia, Uppsala, Sweden
Reverse phase HPLC Column Aquapore RP-300 (C8)	Applied Biosystems (AB1)
(2.1 x 220 mm)	Foster City,USA
UV detector for SMART	Applied Biosystems, Foster
μ Peak detector	City, USA
PTH aminoacid analyzer model 120A	USA
Film developing machine model Gevamatic 60	Agfa-Gevaert, Leverkusen
Heating block thermostat 5320	Eppendorf, Hamburg
Liquid scintillation counter model 1900 TR	Packard, Frankfurt
Confocal laser scanning microscope	Zeiss, Oberkochen
Phosphorimager, IPR 1000	Fuji,Tokyo, Japan
Pipetman 20,200,1000μl	Gilson Medical Electronics, Villiersle-Bel, France
Spectrophotometer,UV 160A	Schimadzu,Kyoto, Japan
Vacuum concentrator speed vac. SVC 100H	Savant instruments,Farmingdale

2.1b Chemicals:

Acetone	Merck
Acetonitrile, HPLC	Baker
30% Acrylamide/ 0.8% Bisacrylamide	Roth
(NN' methylenebisacrylamide)	
Acetic acid	Merck
Ammonium acetate	Fluka
BCA	Sigma
BSA	Biomol, Hamburg
Bromophenol blue	BioRad
Calcium chloride ($CaCl_2$	Merck
Chloroform	Merck
Dimethylsulfoxide ultra pure (DMSO	Merck
PPO	Roth

DTT	Serva
EDTA	Merck
Ethanol	Merck
Ethanolamine	Sigma
Formaldehyde (37%)	Merck
Freund's complete/ incomplete adjuvant	Sigma
Glucose 6-phosphate	Sigma
Glycerin	Merck
Glycine	Roth
Guanidium hydrochloride	Fluka
HPLC grade water	Baker
HEPES	Serva
Imidazole	Merck
Iodoaceticacid (IAA)	Serva, Heidelburg
Iodogen	Pierce Co.
Liquid Scintillation fluid	Roth
p- M.E	Sigma
Methanol	Merck
Mowiol	Calbiochem
Nonidet-40 (NP 40)	Sigma, Deisenhofen
Pansorbin cell suspension	Calbiochem
(Heat inactivated *staphylococcus aureus* cell suspension)	
Paraformaldehyde	Sigma
Phenylmethylsulfonylfluoride (PMSF)	Serva, Heidelburg
Prestained high molecular weight protein markers	Calbiochem
Propylgallat (3,4,5-Trihydroxybenzoic acid propylester)	Serva
Saponin	Sigma, Deisenhofen
Sepharose 6B	Pharmacia
SDS	Sigma
TEMED	Sigma
TCA	Merck
TFA	Fluka
Tris-(hydroxymethyl)-aminoethane (Tris)	Roth
Triton X-100	Sigma, Deisenhofen
Tween 20	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 KC1 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 100^0C 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 $CaCl_2$ and 1 mM $NaHCO_3$
- 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 $CaCl_2$ and 1 mM $NaHCO_3$. 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

-80^0C **until use.**

2.2.4 Extraction of Membrane Proteins and Purification of MPRs:

Note: All operations mentioned below were performed at 4^0C.

Buffer used:

- **Bufferl:** 50 mM imidazole-HCl pH 5.0, 150 mM NaCl, 0.5 mM $CaCl_2$ and 0.1 mM PMSF
- **Buffer 2:** 50 mM Sodium acetate pH 4.6, 150 mM NaCl, 0.5 mM $CaCl_2$
- **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 $MnCl_2$
- **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 $MnCl_2$ (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% $CuSO_4$.

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.

% of PAA gel	6%	7.5%	10%
Acrylamide (ml)	6.3	7.9	10.5
Resolving gel buffer (ml)		7.5	
APS (µl)		250	
TEMED (µl)		25	
H_2O		adj. 30 ml	

Table 4: Reagents used for the preparation of resolving gel with different polyacrylamide concentration

Reagents	Volume
Acrylamide (ml)	1.3
Stacking gel buffer (ml)	2.5
APS (µl)	100
TEMED (µl)	10
H_2O (ml)	6.1

Table 5: Reagents used for the stacking gel

2.2.8 Silver Staining: Ansorge (1985)

Methanol, $CuCl_2 .2 H_2O$, TCA, $KMnO_4$, $AgNO_3$, K_2CO_3, Formaldehyde 37%.

Solutions for the big gel:

1. Fixative: 75 ml methanol, 18 g TCA, 3g $CuCl_2$ 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 $KMnO_4$ in 150 ml water (freshly prepared)

4. 150 mg $AgNO_3$ in 150 ml water (freshly prepared, store in brown bottle)

5. 10%ethanol

6. 10 g K_2CO_3 in 200 ml water (freshly prepared)

7. 60 ml 5% K_2CO_3 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.

Reagent	Big gel	Mini gel
Solution A	15 min	15 min
$KMnO_4$	15 min	10 min
Water (300 ml)	1-2 min	1-2 min
Solution A	15 min	15 min
10%ethanol	15 min	10 min
Water	15 min	10 min
$AgNO_3$	30 min	10 min
Water	1-2 min	1-2 min
K2CO3	2 min	2 min
Developing with solution 7	5-10 min	5-10 min

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 3 rd injection and the blood was allowed to clot, serum was separated by centrifugation and stored at -20^{0}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 (l,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 μl of dichloromethane. 200 μl of it was transferred to another tube and diluted 1-3 times (400 μl) 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 μl 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 μl 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}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 $Na^{125}I$). 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 y-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 95^0C 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 raM NaCl, 5 mM sodium (3-glycerophosphate, 10 mM $MnCl_2$ 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 |il 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 $MnCl_2$

Buffer B: 50 mM imidazole, 150 mM NaCl, 5 mM sodium P-Glycerophosphate, 0.05% Triton X-100, 10 µg/ ml BSA, 10 mM $MnCl_2$ (Table 7)

Column number	PH	Buffer
1	4.0	A
2	4.5	A
3	5.0	A
4	5.5	A
5	6.0	B
6	6.5	B
7	7.0	B
8	7.5	B

Table 7: Column buffers with varied pH used for equilibration of PM gel

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 MSCl 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% CO_2 (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) ^{35}S 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% CO_2 incubator, they were metabolically labeled for 12-14 hours with ^{35}S 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 15min. 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 KC1 in wash immunomix

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

Neufeld buffer: 0.05% (w/v) NP-40, 0.1% (w/v) SDS, 0.6M 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 4^0C overnight, centrifuged at 109,000 x 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 ofantiserum 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 KC1 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 H_2O, sterile filtered.

2 PBS.

3 Ca-Mg-PBS: 0.9 mM $CaCl_2$+ 0.5 mM $MgCl_2$ 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 NH_4Cl in PBS.

6 0.1 % Saponin in PBS (5% stock prepared and stored in aliquots at -20°C).

7 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.

8 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.

9 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% CO_2 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 NH_4Cl, 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 1°Ab. 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 4^0C 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
Iodoaceticacid:	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/ H_2O (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,

- 6 μl 0.4 M Iodoacetic acid, under Argon, incubation at RT in the dark (1AA is light sensitive)
- 6 μl of 0.2 M DTT / H_2O, under Argon, incubation at 50-55°C for 60 min, cooled to RT.
- 6 u1 of 0.4 M IAA, under Argon, incubation at RT for 30 min in the dark.
- 6 μl of 0.2 M DTT / H_2O 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 H_2O

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 ul / 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-Affigel 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.

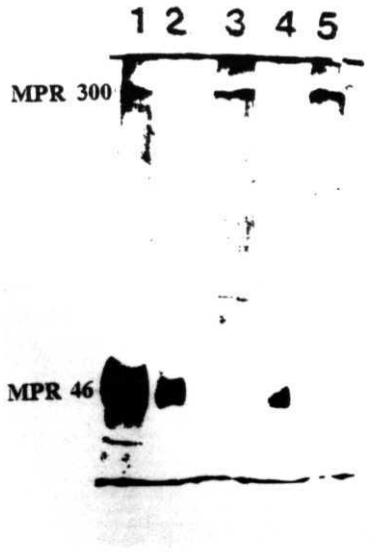


Figure 6

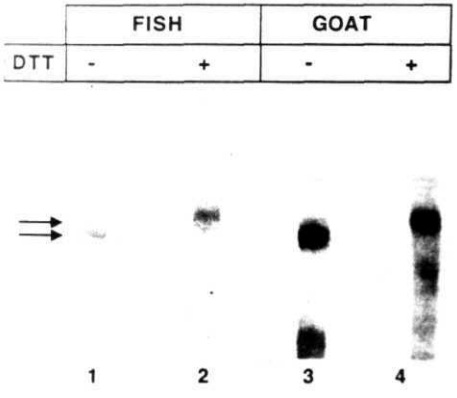


Figure 7

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 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.

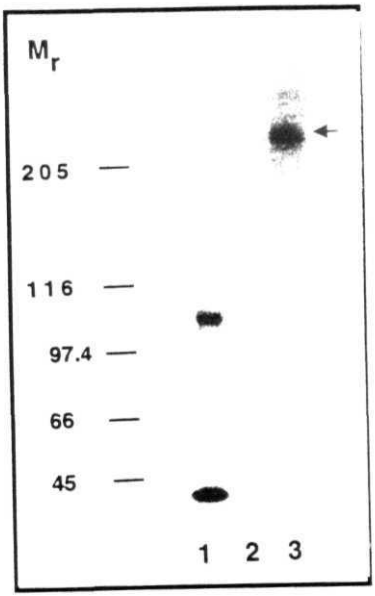


Figure 8

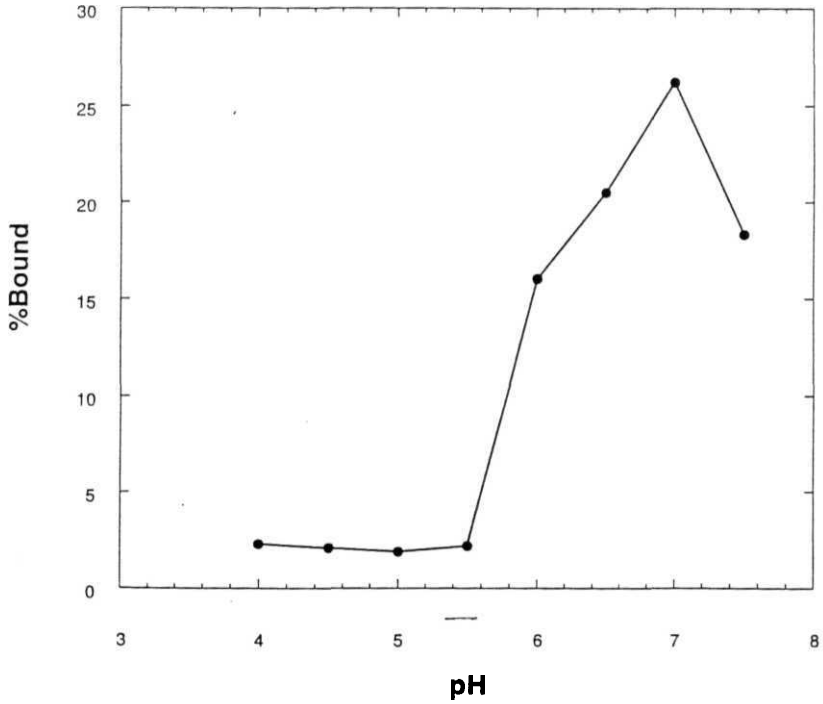


Figure 9

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-MSC1 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.

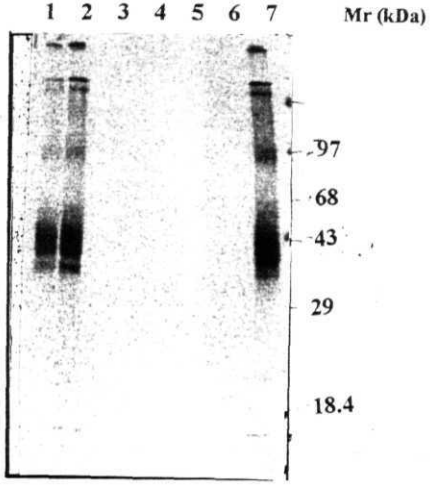


Figure 10

Chicken MPR 46

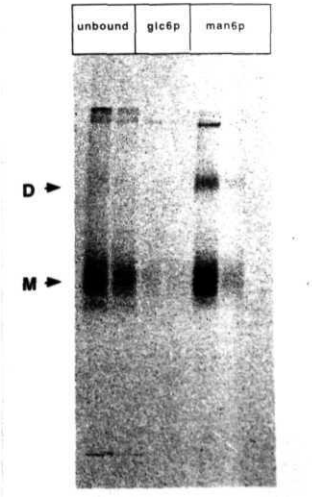


Figure 11

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)**.

Buffer composition	Condition	Temperature	PH	Result
1. 50 mM imidazole, 5 mM sodium β-glycerophospahte (buffer A), 150 mM NaCl, 10 Mm $MgCl_2$	Standard buffer condition	Room temperature/ 4^0C	6.5/7.0/7.5	Strong binding of MPR 300, but no binding of MPR46
2. Buffer A with 90 mM NaCl, each of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$	All the three metal ions included and ionic strength was maintained constant by reducing NaCl to 90 mM	Room temperature/ 4°C	6.5/ 7.0/7.5	No binding
3. Buffer A with150 mM NaCl	In the absence of metal ions	4^0C	7.0	No binding
4. Buffer A with increasing concentration of $MgCl_2$	At an increasing concentration of metal ions 2 mM $MgCl_2$, 5 mM $MgCl_2$ 10 mM $MgCl_2$	4^0C	7.0, 7.0, 6.5	No binding
5. Buffer A with 150 mM NaCl, 10 mM $MgCl_2$	Rebinding of the unbound to a fresh column to see if there is any competitive binding between MPR 300 and MPR 46	4^0C	6.0/6.5/ 7.0	No binding
6. Buffer A with each of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$	NaCl completely omitted and all the three metal ions included	4^0C	6.0/6.5/7.0/ 7.5	Binding of MPR 46 detected

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 $MgCl_2$, 10 mM $MnCl_2$, 10 mM $CaCl_2$ and 5 mM sodium β-glycerophosphate. Each experiment was repeated twice and the mean of two experiments is indicated in the **Table 9.**

	MPR 46 in Column Fractions (% Total)		
pH	**Flow through**	**Glucose 6-p Eluate**	**Mannose 6-p Eluate**
6.0	44.5%	1.2%	54.3%
6.5	31.3%	5.3%	63.4%
7.0	39.9%	9.45%	50.45%

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

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 $MgCl_2$, $MnCl_2$,$CaCl_2$ 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-MSCl 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:

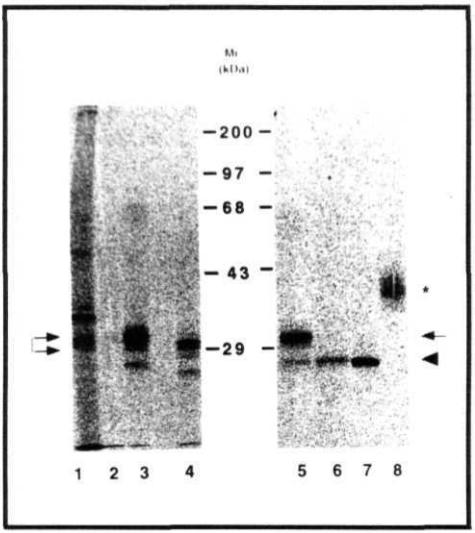
Membrane proteins from *Unio* whole animal tissue acetone powder were extracted as described under methods. The clear membrane extract was passed through PM gel equilibrated with the column buffer, 50 mM imidazole-HCl pH7.0, 150 mM NaCl, 5 mM sodium p-glycerophosphate, 0.05% Triton X-100 and 10 mM $MnCl_2$. 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.

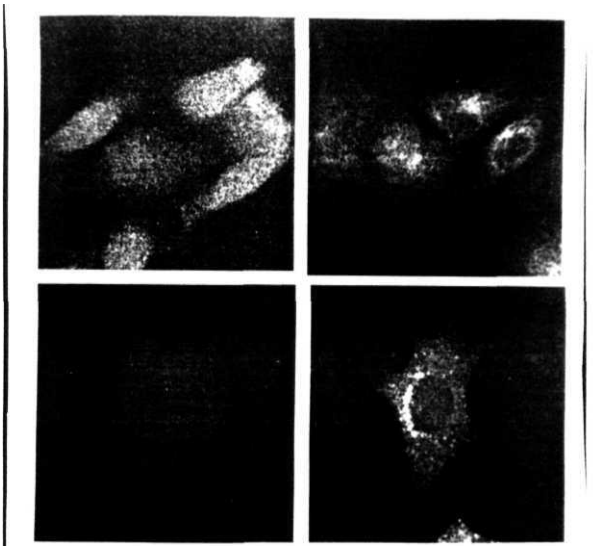
(B) Deglycosylation of fish (lanes 5 and 6) and chicken (lanes 7 and 8) MPR 46 by PNGase F: MPR 46 was isolated by PM-Sepharose chromatography and was immunoprecipitated with anti-MSCl antibody. The immunoprecipitate was split into two equal aliquots and treated for 16 h with (lanes 6 and 7) or without (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-MSCl 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.



A **B**

Figure 12



Fish MPR46

BHK-hu.MPR 46

Figure 13

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 C_8 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.

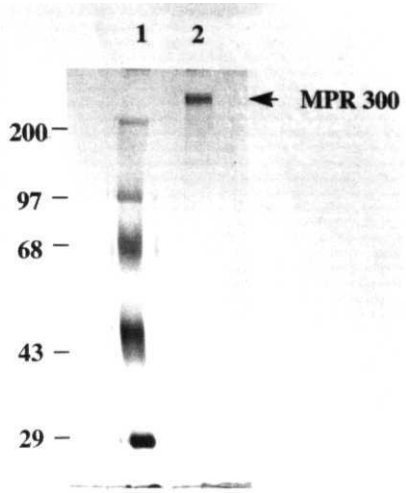


Figure 14

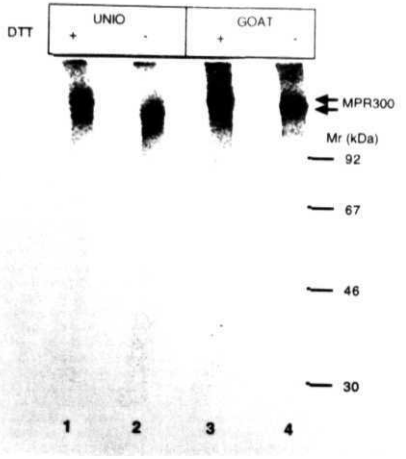


Figure 15

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.

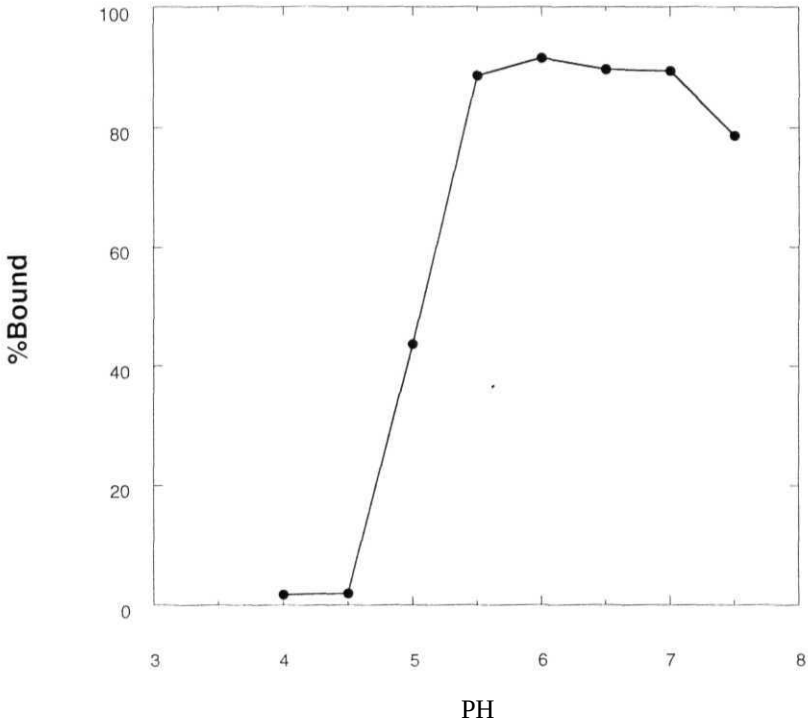


Figure 16

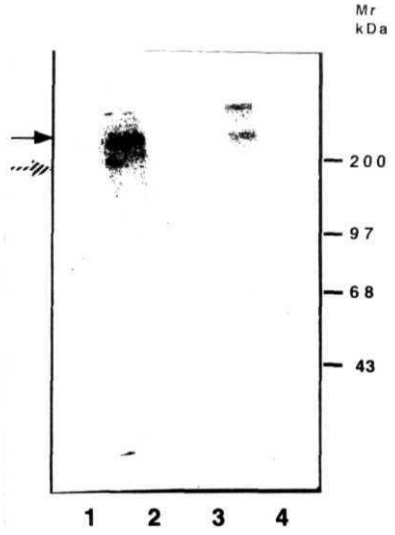


Figure 17

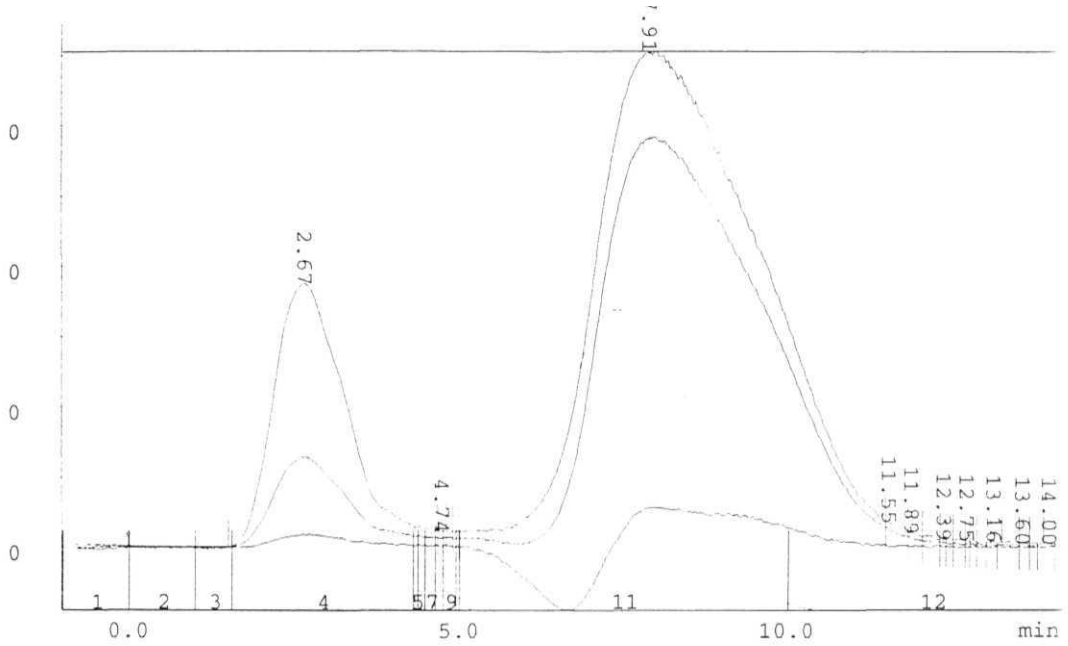


Figure 18: Gel filtration chromatogram of Unio MPR 300 for the separation of reductive carboxy methylated Unio MPR 300 protein fraction from the salt fraction

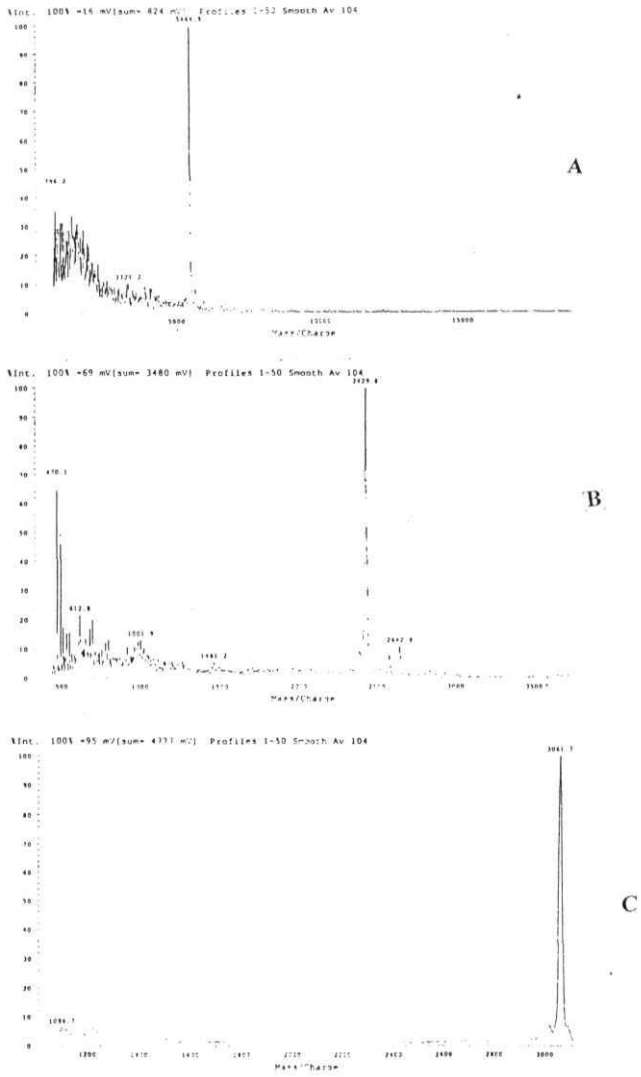


Figure 19: Mass Spectrometry of *Unio* MPR 300 Tryptic Peptides. The affinity purified Unio MPR 300 was subjected to tryptic digestion and the peptides were separated on reverse phase HPLC. The separated peptides were analyzed by mass spectrometry (thin film method). The three peptides shown in the figure were used for sequencing. (A) Peptide 1; (B) Peptide 2; (C) Peptide 3.

Peptide No./Species	Amino acid number corresponding to human MPR 300 total protein
Peptide 1	**146 156**
MPR 300 U	**TLGTPEFV (V/T) AT**
MPR 300 **C**	**TLGTPEFV T AT**
MPR 300 **B**	**TLGTPEFV T AT**
MPR 300 **M**	**TLGTPEFV T AT**
MPR 300 **H**	**TLGTPEFV T AT**
Peptide 2	**1072 1084**
MPR300U	**FLHQDIDS(S)LGIR**
MPR300C	FVRE BINS MLNIH
MPR300B	**FLHQDIDS S LGIR**
MPR300M	**FLHQDIDS** TR**GIR**
MPR300H	**FLHQDIDS** GQ**GIR**
Peptide 3	**1750 1764**
MPR 300 U	**VAGP (P/R) I LN (P) IAN ? VY**
MPR 300 **C**	**VTEP P KLN E** AV**N**E**VY**
MPR 300 **B**	**VAGP P I LN P IAN EVY**
MPR 300 **M**	**VTGP P** I FN **P** V**A**N**EVY**
MPR 300 **H**	**VAGP P I LN P IAN** E**I**Y

*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 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.*,\ 999).

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 *et al*., 1996; for review see Pohlmann, 1996; Siva kumar *et al*., 1997). The MPR proteins were purified from various species by affinity chromatography using immobilized phosphomannan/ pentamannosylphosphate/ *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 *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 E-Affigel that has been used earlier to separate the mixture of the two MPR proteins (Hoflack and Kornfeld., 1985; Stein *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 $MgCl_2$, $MnCl_2$, $CaCl_2$ 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.

CHAPER III

MOLECULAR CLONING AND cDNA SEQUENCING OF FISH MPRs

3.0 INTRODUCTION

The existence of two homologous mannose 6-phosphate receptors with overlapping, but distinct functions has raised a question at what stage in the phylogenetic tree the two receptors have occurred for the first time. The cDNA sequence of mammalian and chicken MPR 300 has revealed that it contains three distinct domains extracytoplasmic (or luminal), transmembrane and cytoplasmic domain. The extracytoplasmic domain is built by 15 repetitive units which share significant sequence similarity with each other and also with the single unit that constitutes the extracytoplasmic domain of MPR 46. In view of these structural similarities and the difference in physiological functions, it is of special interest to identify their common ancestor in the wide spectrum of evolution and to determine at what stage in evolution MPR 300 has acquired its complex repetitive structure.

It is well established that the mammalian MPR 300 is a multifunctional receptor with high affinity binding site for mannose 6-phosphate in the repeat 3 and 9 (Dahms *et al.,* 1993; Westlund *et al.,* 1991) and an additional high affinity binding site for insulin like growth factor-II (IGF-II) in the repeat 11 (Schmidt *et al.,* 1995b). In contrast, chicken and frog MPR 300 fail to bind IGF-II (Clairmont and Czech, 1989; Canfield and Kornfeld, 1989). Binding and endocytosis of IGF-11 by MPR 300 contributes to the regulation of embryonic development in mice. Recently it was also reported that mammalian MPR 300 could also bind retinoic acid (Kang *et al.,* 1997) and human DNAse I (Cacia *et al.*, 1998), but the physiological significance of these properties is not known yet. In the present study, with the use of molecular biological methods and the current knowledge of mammalian and chicken MPR cDNA sequences, we tried to fish out the MPR cDNA sequences from non-mammalian vertebrates and compare the same to the mammalian receptors.

3.1 MATERIALS

3.1a Instruments.

Thermocycler Gene Amp PCR 2400 machine	Perkin-Elmer Cetus, Nowalk, USA
Transilluminator Model IL-400-M	Bachofer, Reutlingen
UV-Hand lamp (312 nm and 254 nm)	Bachofer, Reutlingen
373 A DNA sequencing system	Applied Biosystems

3.1b Chemicals:

Agar	Sigma
Agarose (Electrophoresis gradc)	GIBCO/ BRL
Ammonium acetate	Fluka
Bacto Yeast extract	Difco
Bacto Tryptone	Difco
Dextransulfate	Pharmacia
Diethyl pyrocarbonate	Sigma
Ethidiumbromide	Serva
Formamide	Fluka
p-Formaldehyde	Merck

3.1c Kits Used for Molecular Biological Work:

• QIAgen plasmid mini kit	Diagen, Hilden
• QIAgen plasmid midi kit	Diagen, Hilden
• QIAquick gel extraction kit	Diagen, Hilden
• TA cloning kit	Invitrogen, Groningen, The Netherlands
• Hot star *Taq* polymerase PCR QIAgen kit	Diagen, Hilden
• Dye terminator cycle sequencing kit	Applied Biosystems
• Random primer DNA labeling kit	Amersham, Braunschweig
• QIAgen Phage DNA isolation kit	Diagen, Hilden
• QIAgen RNeasy total RNA isolation kit	Diagen,Hilden
• First strand cDNA synthesis kit	Amersham Pharmacia Biotech, Freiburg, Germany

3.1d Enzymes for Molecular Biological Work:

Alkaline phosphatase	Sigma, Deisenhofen
T_4 DNA ligase	New England Biolabs
Restriction enzymes	New England Biolabs

3.1c **Plasmid DNA Vectors:**

pGem 3zf +/-

pCR 2.1 TA cloning vector

3.1f DNA Standards:

DNA-Ladder	GIBCO/BRL, Eggenstein.
X DNA/Hind-III standard	GIBCO/BRL, Eggenstein.

3.1g **Reagents** Used **for Molecular Biology Work:**

All the following reagents were prepared according to Sambrook *et al.,* 1989.

50x TAE:	2 M Tris-Base
	0.1 M EDTA, pH adjusted to 8.0 with acetic acid.
TE:	10 mM Tris-HCl pH 7.5
	1 mM EDTA.
SO x Denhardt Solution:	5 g Ficoll
	5 g Polyvinylpyrrolidine
	5 g BSA
	Volume was adjusted to 500 ml with deionized water.
1 x SM Buffer:	5.8 g of NaCl
	2.0 g of $MgSO_4 .7H_2O$
	50 ml of 1M Tris-HCl (pH 7.5)
	5.0 ml of 2% (w/v) gelatin
	Volume made up to 1 liter with deionized water, autoclaved and used.

LB medium: 10 g of NaCl
10 g of Tryptone
5 g of Yeast extract, pH adjusted to 7.0 with 5 N NaOH.
Final volume made up to 1 liter with deionized water, autoclaved and used.

LB agar (per liter): 10 g of NaCl
10 g of Tryptone
5 g of Yeast extract
20 g of agar
pH adjusted to 7.0 with 5 N NaOH, final volume made up to 1 liter with deionized water, autoclaved and poured into petri dishes (25 ml/10 cm plate or 60 ml/ 14.5 cm plate).

LB TOP agar: 0.7% agarose in LB medium
Autoclaved and used.

20x SSC Buffer- 175.3 g of NaCl
S8.2 g of Sodium citrate
800 ml of deionized H_2O. Adjust the pH to 7.0 with a few drops of 10 N NaOH, deionized H_2O added to a final volume of 1 liter.

Preparation of Antibiotics: Ampicillin
25 mg/ ml stock solution of the sodium salt of Ampicillin in water; pH adjusted to 8.0 with 2 N NaOH. Sterile filtered and stored in aliquots at -20°C.

Preparation of LB-Agar Plates with Appropriate Antibiotic: LB-Agar autoclaved and cooled to 55°C. Ampicillin added to final 200 µg / ml concentration and poured into petri plates.

Denaturation Buffer: 1.5 M NaCl, 0.5 M NaOH
81.6 g NaCl and 20 g of NaOH dissolved in deionized water and the final volume made up to 1 liter.

Neutralization Buffer:	1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA 10x Tris-buffer (100 ml) was prepared, NaCl and EDTA (pH 8.0) added to a final 1.5 M and 1 mM concentration respectively, volume adjusted to 1 liter.
High Stringency	
Prehybridization Buffer:	48% Formamide 4.8x SSC 10 mM Tris-HCl pH 7.4 1%SDS 1 % Denhardt solution 10% Dextran sulfate. 100 µg/ ml Salmon sperm DNA. Stored at **4°C.**
Low Stringency	
Prehybridization Buffer:	35% Formamide 6x SSC 1%SDS 1% Denhardt solution 10% Dextran sulfate 100 ug/ ml Salmon sperm DNA, stored at 4°C.
3 M Sodium acetate:	408.1 g sodium acetate (tri hydrated) dissolved in 800 ml water, pH adjusted to 5.2 with acetic acid, volume made up to 1 liter and autoclaved and used.
Denatured Salmon Sperm DNA:	Salmon sperm DNA (sodium salt) dissolved in water at a concentration of 10 mg / ml. The solution was stirred on a magnetic stirrer for 2-4 hours at room temperature to dissolve DNA. The DNA was passed through a 20-G gauze needle, boiled for 10 min (at 100°C), sonicated for 1-2 minutes and stored at -20°C in small aliquots.
20% SDS:	20 g of Sodium dodecyl sulfate was dissolved in 100 ml water at 65°C and sterile filtered.

10x MOPS Buffer:

20.93 g of MOPS (200 mM)
2.05 g of sodium acetate (50 mM)
1.86 g EDTA (10 mM)
Dissolved in 300 ml DEPC-H_2O, pH adjusted to 7.0 with 2N NaOH in DEPC-H_2O (for 500 ml, 15-16 ml of NaOH required), after each step of addition of base, pH was controlled by taking an aliquot in an Eppendorf tube, final volume made up to 500 ml with DEPC-water.

5x Loading Buffer:

16 µl Saturated bromophenol blue
80 µl 500 mM EDTA, pH 8.0
720 µl 37% (=12.3 M) formaldehyde
2 ml 100%glycerol
3.084 ml Formamide
4 ml 10x MOPS buffer
RNase-free water added to 10 ml.

SOC medium (pH 7.0):

2.0 g Bacto-Tryptone
0.5 g Bacto-Yeast Extract
1 ml of sterile filtered 2 M Mg^{2+} stock (1 M $MgCl_2.6H_2O$/ 1 M $MgSO_4.7H_2O$), 1 ml of sterile filtered 2M glucose.
Tryptone, yeast extract, NaCl and KC1 were added to 97 ml of deionized water. Stirred to dissolve. Autoclaved and cooled to room temperature. Mg^{2+} stock and glucose were added to a final concentration of 20 mM. Volume made up to 100 ml with sterile deionized water. Filter the complete medium through a 0.2 urn filter unit.

IPTG (isopropylthiogalactoside) stock solution (0.1M):

240 mg of IPTG dissolved in 10 ml of deionized water. Sterile filtered and stored at 4°C.

X-Gal:

100 mg of 5-bromo-4-chloro-3-indolyl-P-D-galactoside dissolved in N,N'-dimethyl formamide, covered with aluminum foil and stored at -20°C.

3.2. METHODS

3.2.1 Total RNA Isolation Using QIAgen Kit:

3.2.1a Important points to be considered while handling RNA:

Glassware: Glassware should be treated as follows before use to ensure that it is RNase free. Fill the glassware with 1N NaOH and leave it overnight or a minimum of l-2hours, rinse with Millipore water followed by 0.1% DEPC in water (*Diethyl pyrocarbonate-carcinogen) and then autoclaved at 100^0C for 15min to remove residual DEPC.

Nondisposable Plasticware: Plasticware should be incubated overnight in 1 N NaOH, thoroughly rinsed with Millipore water and then with RNase free water to ensure that it is RNase free.

Solutions: Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute inhibitor of RNases. It is commonly used at a concentration of 0.1% **to** inactivate RNases on glass or plasticware or to create RNase free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated, shake vigorously to bring the DEPC into solution, and let the solution stand for 12 h at 37^0C. Autoclave for 15 min to remove any traces of DEPC.

3.2.1b Total RNA Isolation from Cultured Cells:

***Xiphophorus* Fish Cell Culture:** *Xiphophorus* A2 (Kuhn *et al*, 1979) embryonic cells were cultured in HAMs F12 medium containing 10% fetal calf serum at 28°C in 95% air / 5% CO_2. Cells were grown to confluence in monolayers in 10 cm plates.

HepG2 Human Hepatoma Cell Culture: HepG2 cells were cultured in DMEM as described (Stoorvogel ***et al.***, 1989), at 37°C in 95% air/ 5% CO_2 incubator and 10% fetal calf serum, 100 U/ ml pen/strep, 2 mM L-glutamine were included as supplements in the growth medium. Cells were grown to confluence in monolayers in 10 cm plates. Cells were split by trypsin treatment (0.05% trypsin).

Counting of Cells: Cells were washed with PBS and were split with 0.5 ml of 0.02% EDTA or 0.05% trypsin and re-suspended in 10 ml PBS. Few ul of the suspension taken for counting in Neubauer chamber. Cells were counted in 16 squares (4 x 4) at 2 different regions and average was taken.

No of cells per 16 squares x 10^4 = number of cells per ml.

*1 x 10^7 cells were used for each preparation as recommended in QIAgen kit.

Harvesting of cells and Preparation of Starting Material: Cells grown on 10 cm plates were washed twice with 1ml of PBS. Cells were harvested (2 x 1ml PBS) with a sterile disposable rubber spatula and transferred to a sterile tube, centrifuged at 250 x g (1000 rpm) for 5 min (Heraeus). Supernatant was aspirated and the pellet recentrifuged as above to ensure complete removal of all liquid.

*Cell pellets can be stored at -70°C for up to 1 year without any degradation.

3.2.1c Total RNA Isolation:

- **Lysis of cells:** Cell pellet was loosened by flicking the tube. Cells were lysed in 0.6 ml lysis buffer (Buffer RLT containing highly denaturing GITC (guanidinium isothiocyanate) which immediately inactivates RNase to ensure isolation of intact RNA) containing 6 ul of P-M.E that was added to the buffer before use.

- **Homogenization:** A homogenous suspension of the cell pellet was made by pipetting up and down and passing 5 times through a 20-G (ϕ 0.9 mm) needle fitted to a syringe.
- 600 µl (1 volume) of 70% ethanol was added (to provide appropriate binding conditions) to the homogenized lysate and mixed well by pipetting up and down (A precipitate may form after the addition of ethanol).
- 700 ul of sample was applied onto RNeasy spin column sitting in a 2 ml collection tube, centrifuged for 15 seconds at 13,000 rpm (Eppendorf centrifuge).
- The above step was repeated for rest of the sample.
- The Column was washed with 700 ul of wash buffer RWl by applying the buffer on column and centrifuging for 15 seconds at 13,000 rpm.
- Flow through and collection tube were discarded.
- RNeasy spin column was transferred to a new 2 ml collection tube and washed as above with 500 µl of buffer RPE.
- Column was rewashed with 500 ul of buffer RPE, centrifuged for 2 min at 13,000 rpm to dry the RNeasy membrane (*It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent elution).
- Column transferred into a new sterile 1.5 mi collection tube (supplied). 50 ul of RNase free water was pipetted directly onto the membrane, allowed to stand for a minute, centrifuged for 1 min at 10,000 rpm to elute RNA.

3.2.2 **Quantitation of Nucleic acids (RNA / DNA):**

The concentration and purity of RNA/DNA was determined by measuring absorbency at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer.

* A_{260}=1 corresponds to 40 μg/ ml of RNA or 50 μg/ ml double stranded DNA. The ratio between the absorbency readings at 260 nm and 280 nm gives an estimate of purity. Pure RNA/DNA preparation will have an A_{260}/A_{280} ratio of 2.0.

3.2.3 Denaturing Agarose Gel Electrophoresis for RNA:

1%Agarose gel:

Agarose (electrophoresis grade)	2 g
1 Ox MOPS-buffer	20 ml
DEPC-water	180 ml

Boiled in microwave, cooled to 65^0C, 20 μl of EtBr (10 mg/ ml) and 3.3 ml of formaldehyde (37%) added, mixed well and poured into the sealed electrophoresis trough fitted with a comb. Solidified gel was cut to the required size, placed in the electrophoresis chamber.

Sample Preparation and Gel Run:

RNA sample (1-5 μg) was mixed with loading buffer (1 vol. of sample buffer to 4 vol. of RNA sample), cooked at 65°C for 5 min, chilled on ice and used for loading. Gel was run at 70 V for 3 hours.

3.2.4 Agarose Gel Electrophoresis for DNA:

DNA fragments were subjected to agarose gel electrophoresis for resolution. Based on the size **of** DNA fragments to be resolved, the percentage of the gel is varied as shown below in the **Table** 10.

Agarose Concentration (% (w/v))	Range of Fragments Size(kb)
0.7	10-1.0
1.0	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

Table 10: Percentage of Agarose gel to be used for the Separation of DNA Fragments of **Different Lengths**

Sample buffer (Loading buffer IV)	0.25 %(w/v) Bromophenolblue
	40 % (w/v) Saccharose in TAB
Ficoll-Marker	0.05% (w/v) Bromophenolblue
	0.05% (w/v) Xylenecyanol
	15% (w/v) Ficoll

Depending on the percentage of the gel, agarose was weighed and added to 300 ml of TAE, cooked in microwave and cooled to 55^0C. Ethidium bromide was added to final 0.5 µg/ ml, mixed and poured into the gel trough fitted with the combs, allowed to cool to room temperature. The gel was cut into the required size with required number of wells. Sample was mixed with sample buffer (10-20% (v/v)) and loaded in the wells, gel was run for 1 to 2 hours in 1 x TAE buffer at 120-240 V depending on the size. The gel was viewed under UV transilluminator.

3.2.5 Gel Documentation:

Nucleic acids intercalated with the fluorescent dye ethidium bromide was visualized under UV light using transilluminator and the print out was taken.

3.2.6 Primer Designing:

The parameters to be considered during primer selection are,

- **Length:** 18-30 nucleotides.
- G/C **Content:** 40-60%
- **T_m:** T_m= **2°C** x **(A+T)** + **4°C** x **(G+C).** If possible design primer pairs with a difference of + 2°C Tm values. Optimal annealing temperature may be calculated as 5°C below the estimated melting temperature.
- Complementarity of two or three bases at the 3'ends of primer pairs was avoided to reduce primer dimer formation.
- Complementary sequences within a primer sequence was avoided to reduce hairpin formation.
- Primer with A or T at 3' end is avoided, as it has greater tolerance of mismatch. It is always advantageous to have G/C at the 3' end.
- The compute program ,,Oligo analysis" was used for primer designing.

  Once the primers were selected, blast search was done to see percentage homology with thc known sequences.

3.2.7 Reverse Transcription or First Strand cDNA Synthesis (Pharmacia kit):

First strand cDNA synthesis is catalyzed by Moloney Murine Leukemia Virus reverse transcriptase. The readymade bulk first strand cDNA synthesis reaction mix requires only the addition of RNA, DTT (dithiothreitol), and a primer of choice.

3.2.7a First-Strand cDNA Synthesis:

- 5 µg (in 8 u.l) of total RNA isolated was taken in a sterile eppendorf tube, heat denatured at 65°C for 10 min, then chilled on ice.
- 5 u.l of Bulk strand reaction mix (Bulk strand reaction mix must be gently pipetted up and down to obtain uniform suspension) taken in a sterile eppendorf tube. To this tube 1 µl of DTT (200 mM) solution, 1 µl (40 pmoles) of specific anti-sense primer and the heat denatured RNA were added (see **Table 11).**

Solution/ reagent	Final concentration/ µl	Total amount used/15µl reaction
RNA	0.3 µg	5 µg
Bulk strand reaction mix	_____	5 µl
Anti-sense primer	2.67 pmol	40 pmol
DTT	13.3 mM	1µl (200 mM)

Table 11: Composition of 1st Strand Synthesis Assay

- Above reaction mix was incubated at 37^0C for 1 hour.
- The completed first strand reaction product was heated at 90°C for 5 min (to denature RNA-cDNA duplex and to inactivate the reverse transcriptase) and chilled on ice. The denatured RT product was used for PCR amplification using specific sense and anti-sense primers.

3.2.8 PCR Amplification (QIAGEN Hot Star Taq DNA Polymerase Kit):

Materials:

Hot Star *Taq* DNA Polymerase

Hot Star *Taq* DNA Polymerase is a recombinant 94 kDa *Taq* DNA Polymerase. Hot Star *Taq* DNA Polymerase is provided in inactive state with no polymerase activity at ambient temperatures. This prevents the formation of misprimed products and primer-dimers at low temperatures. Hot Star *Taq* Polymerase is activated by a 15 min incubation at 95 C. Hot Star *Taq* DNA Polymerase provides high PCR specificity and often increases the yield of the specific PCR product.

Q-Solution

Hot Star *Taq* DNA polymerase is provided with Q-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behaviour of DNA. Q-Solution may have several effects such as (1) May enable amplification of a reaction which previously failed, (2) May increase PCR specificity in certain primer-template system, (3) May have no effect on PCR performance, (4) May cause reduced efficiency or failure of a previously successful amplification reaction. Therefore, always the reactions were performed in parallel with and without Q-Solution whenever used for the first time.

3.2.8a PCR Reaction:

Polymerase chain reaction is a method used to amplify DNA fragments of interest from the template DNA with the use of sense and anti-sense primers of specific interest

Composition of PCR Amplification Assay:

dNTP mix:	Each 10 mM dATP, dCTP, dGTP and dTTP
Hot star *Taq* DNA polymerase:	5 Units/ 1 µl.
1 Ox PCR buffer:	Tris-HCl, KC1, $(NH_4)_2SO_4$, 15mM $MgCl_2$, pH 8.7 (20°C)
Sense and anti-sense primer	each 40 p moles/ µl

Following were used for one PCR reaction:

10 x PCR buffer	10 ul
dNTP Mix	2 ul
Primers	1 ul each of sense and anti-sense primers.
5 x 'Q' solution (with or without)	20 ul
Taq DNA polymerase	0.5 µl
Template DNA	
(First strand cDNA (5 µl) or DNA)	x vol.

Master mix was prepared as above. Template DNA was added at the end, volume was made up to 100 µl with sterile water. The PCR reaction was carried out using the thermal cycler program shown in the **Table 12.** The annealing temperature and others were modified depending on the T_m and specificity of the primers used for amplification.

Reaction Step	Time (min)	Temperature (°C)
Initial activation step	15	95
Repeated Number of Cycles	**30-35**	
Denaturation	1	94
Annealing	45 sec	T_m - 5 to 15
Extension	1	72
Final extension	**10**	**72**
Hold	a	4

Table 12: Thermal Cycler Program

3.2.9 Gel Purification of PCR Product (QIAquick Gel Extraction Kit):

A preparative agarose gel was run, cDNA band of expected length was cut with a clean sharp scalpel. Gel slice was weighed, 3 volumes of buffer QG (solubilization and binding buffer) was added to one volume of gel (100 mg = 100 µl). The maximum amount of gel slice per QIA quick column is 400 mg. Incubated at 50°C for 10 min (or until the gel slice was completely dissolved). During incubation the tube was vortexed for every 2-3 min to dissolve the gel. After the gel slice has dissolved completely, the color of the solution was similar to QG buffer colour.

One volume of isopropanol was added to the sample and mixed. (This step increases the yield of DNA fragments between 500 bp and 4 kb). QIAquick spin column was placed in a 2 ml collection tube. To bind DNA, the sample was applied to the column, and centrifuged for 1 min. (The maximum volume of column reservoir is 800 µl). Flow through was discarded, QIAquick column placed back into the same collection tube. 0.5 ml of buffer QG was added to the column and centrifuged for 1 min (This step will remove all traces of agarose). Column was finally washed with 0.75 ml of buffer PE, by adding the buffer to the column and was centrifuged for 1

min. Flow through was discarded, column was centrifuged for an additional 1 min at 13,000 rpm. Column was placed into a clean 1.5 ml microfuge tube. To elute DNA, 50 ul of sterile water was added to the center of the QIAquick column, allowed to stand for 1 min and centrifuged for 1 min at maximum speed. Eluted DNA was analyzed by analytical agarose gel electrophoresis.

3.2.10 TA Cloning (Invitrogen):

Introduction:

Taq Polymerase has a non template-dependent activity, which adds a single deoxyadenosine (A) **to** the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The kit provides a quick, one step cloning strategy for the direct insertion of a polymerase chain reaction (PCR) product into a plasmid vector **(Fig** 20). Advantages using the kit are, one can eliminate any enzymatic modifications of the PCR product.

Amount of **PCR Product Required for Ligation:**

Amount of PCR product required for cloning into pCR 2.1 vector was estimated using the following formula

X ng PCR product = (Y bp of PCR product) (50 ng pCR 2.1 Vector) /

(Size **in bp of the pCR 2.1 vector : 3,900 bp)**

3.2.10a **Ligation:**

- One vial of pCR 2.1 vector was centrifuged to collect all the liquid in the bottom of the vial.

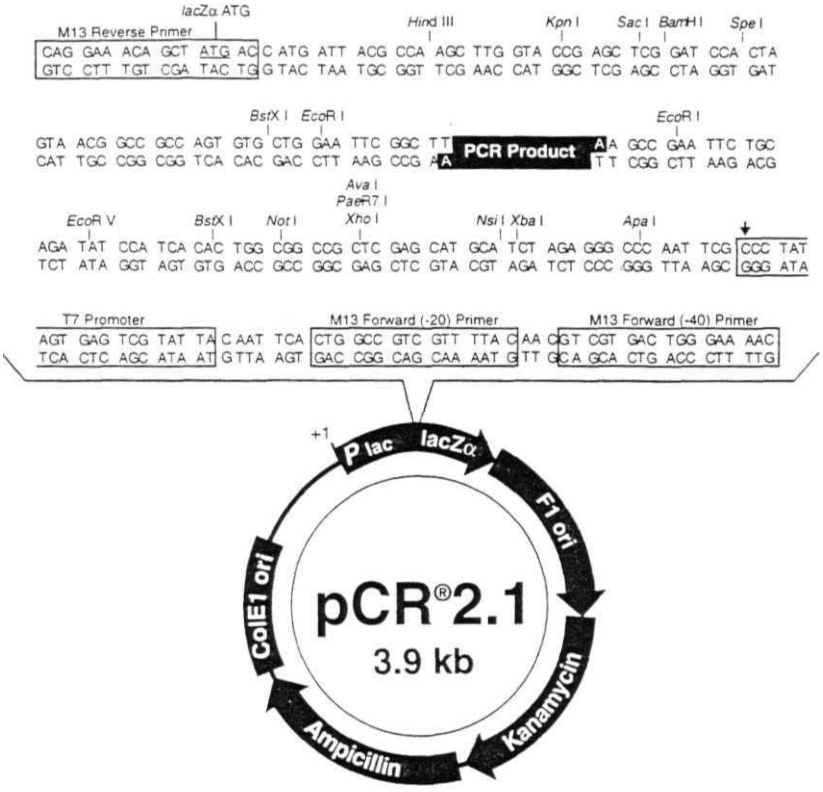


Figure 20: pCR 2.1 Plasmid Vector for TA Cloning. The sequence above represents the pCR 2.1 vector **with** a PCR product inserted by TA cloning which is flanked by EcoRI site on each side. ▼indicates the start of transcription for the T7 RNA polymerase.

- Ligation reaction was set up as follows

Fresh gel purified PCR product	x ul
10 x Ligation buffer	1 ul
pCR 2.1 Vector (50 ng)	2 ul (25 ng/ ul)
Sterile water	1 ul
T_4 DNA Ligase (4.0 Weiss units)	1 ul
Sterile water to final volume	10 µl

- Ligation reaction was carried out at 14^0C (water bath) overnight in the cold room.

Note: The ligated vector can be used for transformation or can be stored at -20^0C until ready for transformation.

3.2.10b Transformation:

Before Start:

- Water bath was equilibrated to 42°C.
- A vial of SOC medium was thawed and incubated at 37°C.

Preparation of agar plates: Ampicillin LB agar plates (two plates for each ligation/ transformation) were equilibrated at 37^0C for 30 minutes. Each plate was coated with 40 ul of 40 mg/ ml X-Gal (chromogenic substrate for β-galactosidase) 60 ul of 100 mM IPTG (isopropylthiogalactoside-used to maximize the expression of genes cloned in expression vectors). Liquid was allowed to soak into the plates by incubating at 37°C for 20-30 min.

* Plates may also be made ahead of time (2-3 hours).

Procedure for Transformation:

The vials containing the ligation reactions were centrifuged briefly to bring the contents down and placed on ice. A vial of 0.5 M β-M.E and one vial of frozen One Shot competent cells (TOP10F' provided in the kit) were thawed on ice for each transformation. 2 ul of 0.5 M p-M.E was pipetted into each vial of the competent cells and mixed by stirring gently with pipette tip (* **Do not mix by pipetting up and down).**

2 µl of each ligation reaction was directly pipetted into the competent cells and mixed by stirring gently with pipette tip. Vials were incubated on ice for 30 minutes. The remaining ligation mixture was stored at - 20^0C. Heat shock was given exactly for 30 seconds in a 42^0C water bath **(*Do not** mix **or shake).** The vials were removed from the water bath and placed on ice for 2 minutes. 250 µl of SOC medium (at room temperature) was added to each tube. The vials were kept for horizontal shaking at 37°C for 1 hour at 225 rpm in a rotary-shaking incubator. The vials with the transformed cells were placed on ice. 50 ul and 200 ul from each of the transformation vial was spread on separate, labeled LB agar plates containing 50 µg/ml of ampicillin, preadsorbed with X-Gal and IPTG. The liquid was allowed to be absorbed, then the plates were inverted and placed in 37°C incubator for atleast 18 hours. Plates were then shifted to cold room for proper colour development.

*The transformed cells appear white in contrast to the untransformed blue colonies.

3.2.11 Plasmid DNA Isolation (QIAprep plasmid DNA Isolation kit):

Bacterial colony picked up with a sterile toothpick and inoculated into 5 ml medium (LB medium containing 200 µg/ ml ampicillin), overnight culture was prepared for plasmid DNA isolation and restriction analysis. 2 ml of overnight culture was taken into a sterile microcentrifuge tube, centrifuged at 6,000 rpm (1800 x g) for 5 min. Supernatant aspirated and pellet was

recentrifuged briefly to remove all liquid. Bacterial cell pellet was resuspended in 250 µl of buffer P1. 250 µl buffer P2 was added and the tube was gently inverted for 4-6 times (solution becomes viscous and slightly clear). 350 µl of buffer N3 was added and the tube was inverted immediately but gently 4-6 times to avoid localized precipitation (solution becomes cloudy), centrifuged at 13,000 rpm for 10 min. Meanwhile QIAprep spin column was placed in a 2 ml collection tube.

The supernatant from the above step was applied to the column, centrifuged briefly, flow through discarded. Column washed by adding 0.5 ml of buffer PB and centrifuged at 13,000 rpm for 30-60 sec, flow-through was discarded. Column was washed by adding 0.75 ml of buffer PE, centrifuged at 13,000 rpm for 60 sec, flow through discarded and centrifuged for an additional 1 min to remove residual wash buffer. QIAprep column was placed in a clean 1.5 ml microfuge tube. 50 µl of sterile water was dropped exactly in the middle, over the membrane, incubated for a minute and centrifuged for a minute at 13,000 rpm for eluting DNA.

3.2.12 Midi preparation of Plasmid DNA (QIAgen method):

The following buffers are supplied in the kit

P1 (Resuspension buffer):	50 mM Tris-HCl pH 8.0
	10 mM EDTA
	100 µg/ ml RNase A
P2 (Lysis buffer):	200 mM NaOH, 1 % SDS
P3 (Neutralization buffer):	3.0 M potassium acetate pH 5.5
QBT (Equilibration buffer):	750 mM NaCl, 50 mM MOPS pH 7.0,
	15% isopropanol, 0.05% Triton X-100
QC (wash buffer):	1.0 M NaCl, 50 mM MOPS pH 7.0
	15% isopropanol

3.2.11 Procedure for Plasmid DNA Isolation:

100 ml of the bacterial culture with an OD_{600} in the range of 1.0 to 1.5 was centrifuged at 8500 x g in a JA 10 rotor (8000 rpm). The supernatant discarded, bacterial pellet was resuspended in 4 ml of buffer P1, transferred to JA 20 centrifuge tube, 4 ml of buffer P2 added mixed by inverting 4-6 times and incubated at room temperature for 5 min. 4 ml of chilled buffer P3 added, mixed immediately but gently by inverting the tube 4-6 times, incubated on ice for 15 min. Centrifuged at > 20,000 x g (13,000 rpm) in a JA 20 rotor for 30 min at 4^0C. The supernatant containing the DNA was carefully transferred to another tube and re-centrifuged as above for 15 min. The supernatant was loaded on the QIAGEN-tip 100 column pre equilibrated with 4 ml of QBT buffer and the sample was allowed to enter the resin by gravity. The column was washed twice each time with 10 ml of buffer QC and finally the bound DNA was eluted with 5 ml of buffer QF into a 50 ml centrifuge tube.

The DNA was precipitated by adding 3.5 ml (0.7 vol) of room temperature isopropanol, mixed and centrifuged immediately at 15,000 x g (11,000 rpm) for 40 min. The supernatant was discarded carefully without touching the pellet. DNA pellet was washed with 2 ml of room temperature 70 % ethanol, and centrifuged at 15,000 x g (11,000 rpm) for 10 min. The supernatant was discarded without disturbing the pellet. Pellet was air-dried for 5-10 min and redissolved in 100 µl of sterile deionized water. DNA concentration was estimated by measuring OD at 260 nm.

3.2.13 Digesting DNA with Restriction Endonucleases:

10 x buffer composition supplied by NEB New England Biolabs with the restriction enzymes:

NEB 1: 50 mM Tri-HCl pH 7.0 + 10 mM $MgCl_2$ + 1 mM DTT

NEB 2: 50 mM Tri-HCl pH 8.0 + 10 mM $MgCl_2$ + 1 mM DTT + 50 mM NaCl

NEB 3: 50 mM Tri-HCl pH 8.0+10 mM $MgCl_2$ + 1 mM DTT + 100 mM NaCl

NEB 4: 50 mM Tri-HCl pH 8.0 + 10 mM $MgCl_2$ + 1 mM DTT + 50 mM KAc

About 0.5 to 1.0 µg of plasmid DNA was used for restriction analysis with restriction enzyme as specified. Volume of the DNA sample was made upto 8 ul to this, 1 µl of enzyme and 1 µl of 10 x buffer was added. Each restriction enzyme has a set of optimal reaction buffer conditions, which are supplied by the manufacturer. The sample was incubated at **37°C** for 1-2 hours. Product was analyzed by analytical agarose gel electrophoresis.

3.2.14 *V* 3' Dideoxy NTP Dye Terminator Cycle Sequencing (Applied Biosystem DNA **Sequencing kit):** Sanger and Coulson (1977).

The nucleotide sequence was determined by cycle sequencing based on the dideoxy nucleotide chain termination method using the dideoxy NTP dye terminator cycle sequencing kit.

Primers: vector specific sequencing primers M13 forward, M13 Reverse Primer, T7, SP6, or MPR specific primers as mentioned (3.5-10 pmol / 2.5 µl).

1 µg of plasmid DNA or 2.5-5u.g of phage DNA used for each reaction.

The following reaction was set up

DNA	Premix	Primer
1 Hg	4 µl	10 pmoles

Volume was made upto 20 µl with sterile water.

Thermo Cycler Program:

1. Denaturation	96^0	C	10 sec
2. Annealing		50^0C	5 sec
3. Primer extension		60°C	4 min

Total number of cycles 25

Purification and Precipitation of DNA:

To the sequencing PCR product, 2 ul of 3 M sodium acetate (pH 5.2) and 50 ul of 100% ethanol were added and centrifuged at high speed for 20 min. Pellet was washed with 250 ul **of** 70% ethanol, centrifuged at maximum speed (14,000 rpm, Eppendorf centrifuge) **for** 10 **min.** Pellets were dried in speed vac for 3 min. Pellets were finally dissolved in 2.8 ul of sample buffer "BIG dye" (supplied in the kit), and electrophoresed and sequenced using 373 A DNA sequencing system according to the manufacturers manual.

3.2.15 Random Primer Labeling (Redivue random primer labeling kit):

25 ng of gel purified DNA probe was dissolved in 45 ul of sterile water. DNA denatured by heating at 95°C for 5 min in boiling water bath followed by chilling on ice. Denatured DNA was added to the labeling mix (contains dATP, dGTP, dTTP, Klenow-Fragment of DNA Polymerase I, random primers), mixed gently by flicking the tube. (a ^{32}P) dCTP (2ul-5 ul (20-50uCi)) offered and incubated at 37°C for 10 min. The reaction was stopped by adding 150ul of TE (10mM Tris-HCl pH 8.0 and 1mM EDTA). Labeled probe was separated from un-incorporated dNTPs by passing over Sephadex G-50 (500 ul) packed in lml blue tip, centrifuged for 2 min at 3000 rpm. Second elution was done with 100 ul of TE buffer. 1/100 of eluted volume was used for cerenkov counting.

% Incorporation

'x' cpm x 100 = Total counts.

Calculate total uCi (1 uCi = 2.22 $x10^6$ cpm)

$$\%\text{Incorporation} = \frac{\text{Total counts obtained (in uCi)}}{\text{Radioactivity offered}}$$

1 uCi / ml of hybridization buffer used.

3.2.16 Northern Blotting (RNA Transfer)/ Southern Blotting (DNA Transfer):

Southern.,(1975)

Pretreatment of agarose gel for DNA transfer:

After the gel electrophoresis, the gel was soaked in the following buffers,

2 times, each with 15 min incubation in denaturing buffer

2 times, each with 15 min incubation in neutralization buffer

10 min in 20 x SSC.

Pretreatment of formaldehyde-agarose gel for RNA transfer:

After the electrophoresis, the gel was soaked in 20 x SSC buffer for 40 min.

Note:

Before assembling the gel for transfer, the gel picture with scale was taken by exposing briefly to UV at higher wavelength (312 nm).

During the transfer, gel was inverted so that the wells are facing down.

During the transfer, sides of the gel were covered with polythene cover (cut into narrow pieces) to prevent the buffer from spreading to the sides

The transfer of nucleicacids from agarose gel to hybond-N nylon membrane was done just by capillary action by assembling the gel for transfer as shown in the **Figure 21.** Overnight transfer was done at room temperature. Filter was air dried, exposed to UV light for 15 seconds, oven baked at 60^0C for 1 hour and used for hybridization.

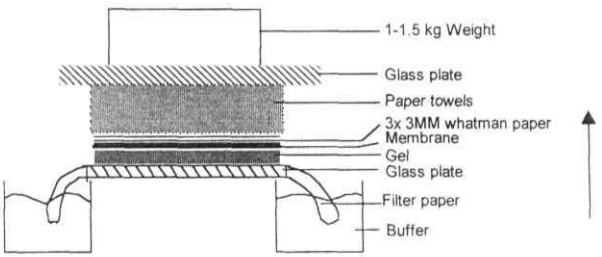


Figure 21: Schematic representation for the assembly of gel for capillary transfer of DNA/ RNA from the gel to membrane. Arrow indicates the flow of the buffer.

3.2.17 Hybridization:

Prehybridization:

The filters or membranes (Northern or Southern blots) were placed in a cylinder, 10-20 ml of prehybridzation solution added according to the number of filters, tightly covered with a rubber cork to prevent any evaporation, and incubated at 42°C with rotation for about 2-3 hours.

Hybridization:

^{32}P labeled DNA probe ($1\text{-}2x10^6$ cpm/ml of hybridization **mix) and** salmon sperm DNA (100 µg / ml of hybridization mix) were denatured by heating for 5 minutes at 100^0C and added to prehybridization mix covering the filters and overnight incubation was done at 42 C with rotation. During the hybridization, the container holding the filters were tightly closed, placed in a polythene bag and sealed to prevent any leakage.

Washing:

Alter the hybridization was completed, the container was placed straight to drain the liquid down, then with **the** help of a forceps, membrane was placed in a 2x SSC buffer facing the DNA/RNA downwards, to prevent drying of the filter. Washing was done as follows

Once with 2 x SSC and 0.1% SDS at room temperature for 10 min.

Once with 2 x SSC and 0.1% **SDS** at 65°C for 30 min.

Radioactivity was controlled, if it was too high, an additional washing was performed with 0.2 x SSC and 0.1% SDS at 65°C.

When the background was still high, further washing was done at high stringency conditions (0.2% SSC and 0.1% SDS) as above. Membrane placed on shining surface of the bench coat paper, covered with saran wrap, exposed to Kodak film (XOMAT AR) overnight at -70^0C with an intensifying screen. Alternatively the membrane was also exposed to phosphorimaging screen and scanned after an overnight exposure.

3.2.18 Long Term Storage of Bacterial Stocks:

Bacteria can be stored for many years in media containing 15% glycerol at low temperatures without significant loss of viability. A single bacterial colony was inoculated into 5-10 ml of **LB** medium taken in a culture flask and the culture was grown overnight. 0.85 ml of the overnight culture was transferred to a sterile vial containing 0.15 ml of sterile glycerol. The contents were mixed thoroughly by vortexing. The glycerinated cultures are then stored at **-20°C** in small aliquots. They can be preserved for few years without loss of viability. Alternatively, the glycerinated suspension was stored at -70°C. Viable bacteria were recovered by simply scratching the surface of the frozen stock with a sterile platinum loop or wire and used **for** streaking on the agar plate or directly inoculated into liquid culture medium.

3.2.19 Titering of Phage Library:

3.2.19a Preparation of the Agar Plates (10cm/14cm):

Agar plates (25 ml LB-agar/10 cm or 70-80 ml LB-agar /14 cm plates) without any antibiotic were prepared well in advance (they should be atleast 2 days old and can be longer also). The plates (placing agar to the surface) were preincubated at 37 C for a minimum of 3 hours.

3.2.19b Preparation of Host Cell (LE392) Culture:

* A single bacterial cell colony (LE392) was inoculated into 50 ml of LB-broth supplemented with 0.2% maltose and 10 mM $MgSO_4$ in a sterile 250 ml Erlenmeyer flask. (Bacteria grown in the presence of maltose adsorb bacteriophage with more efficiency, as it induces the expression of phage receptor, which favors the infection).

- The culture was grown overnight with shaking at 30^0C (This lower temperature ensures that the cells will not overgrow and die, as the phage can adhere to dead cells as well as to live ones resulting in low titer).

* Cells were sedimented at 500 x g for 10 min, the supernatant discarded. Cells were resuspended in 20 ml (0.4 vol. of original culture volume) of 10 mM $MgSO_4$ and absorbency at 600 nm was measured. A proper dilution was done to get 0.5 O.D at 600 nm (usually 10-15 ml final volume).

3.2.19c Dilution of the Phage Library:

Phage dilution of 1:1000 was done in SM buffer, and from this further dilution **of** 10 fold was done in the SM buffer depending on the requirement.

3.2.19d Phage Infection:

50 µl of diluted phage and 50 µl of host cells (A_{600} =2 is $1.5x10^9$cells /ml) were mixed and incubated at 37^0C for 15 min with rotation.

3.2.19e Plating of Bacteria:

The infected cells were mixed with 3 ml of 0.7% top agar equilibrated to 48°C, spread uniformly over the preincubated **(37°C)** agar plates, incubated at 37°C for a minimum of 8-12 hrs. Number of plaques formed were counted after 12-16 hrs of incubation using cell counter.

liter determination: 'X' number of plaques in 'Y' volume x Dilution factor = number of plaques in 'Y' volume of undiluted phage supernatant.

3.2.20 **Screening of the Phage Library:**

3.2.20a Plating of phage infected bacteria:

Total of 1 million pfu (plaque forming units) were used for screening. 50,000 pfu were taken on each plate of 14 cm. A fresh culture of LE392 host cells was prepared, cells were diluted to an $O.D_{600}$ of 0.5 in 10 mM $MgSO_4$. 12 ml (A_{600} = 0.5) of host cells and $1x10^6$ pfu of phage library

were mixed and incubated for 15 min at 37°C. The phage infected cell suspension was divided into 20 aliquots (each aliquot contains 50,000 pfu), each was taken in mini culture glass tubes, each aliquot was mixed with 6.5 ml top agar (0.7% top agar, melted and equilibrated to 48°C) and was spread uniformly over 14 cm bottom agar plates (minimum 2 days old) preheated to **37°C** for a minimum of 4 hours. Plates were incubated at 37^0C for 8-10 hours (do not allow the plaques to get larger than 1-2 mm). Plates were chilled at 4^0C for a minimum of 2-3 hrs to prevent the top agar from sticking to the hybond-N nylon membrane filters.

3.2.20b Plaque Lifting:

*Note: The following steps were performed with forceps and handling was done wearing gloves. Plaque transfer was done to hybond nylon-N membrane (Amersham) filters. Filters and agar plates were numbered as 1-20, filter placed over the plate, plaque transfer was allowed to **take** place for 10 min, filter was pricked into agar with a needle at 3 asymmetric spots for proper orientation, plates were marked with a permanent marker at the bottom of each plate corresponding to pricked spots. When plaque lifting was done in duplicates, the second plaque transfer was done for 15 min. Filters were processed as follows,

3 MM Whatman filter paper was cut into 3 pieces, the first one was prewetted with denaturation buffer, the second and third were prewetted with neutralization buffer. Denaturation and neutralization of membrane filters was done as shown in the **Figure 22.** Membrane filters were denatured by placing the filters on Whatman 1, with plaques facing top, incubated for 10 min. Membrane filters were then shifted to second Whatman paper for neutralization for 10 min and then transferred to the third Whatman paper for a 2nd neutralization step.

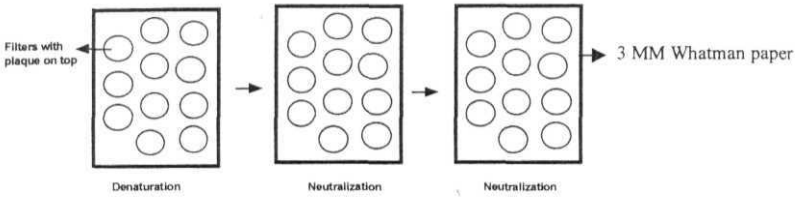


Figure 22: Schematic representation for the processing of filters with denaturation and neutralization buffers

Membrane filters were rinsed briefly (not more than 30 seconds) in 2 x SSC buffer, then blotted on 3 MM Whatman paper. Filters were air dried, DNA was UV cross-linked for 15 seconds, oven baked at 60^0C for 20-30 min, the filters were then wrapped in polythene bag and stored at room temperature until further use. The corresponding plates were sealed with parafilm and stored at 4°C for later use.

3.2.20c Colony Hybridization:

Following the preparation of the membranes for hybridization, membranes were subjected to prehybridization, hybridization as follows.

Prehybridization:

The membrane filters were placed in a cylinder (400ml capacity) with denatured DNA facing towards the wall, 40-50 ml of prehybridzation solution (low or high stringency buffer) added, tightly covered with **a** rubber cork to prevent any evaporation, and incubated at 42^0C **with** rotation for about 2-3 hours. Two cylinders were used taking 10 membranes in each.

Hybridization:

^{32}P labeled DNA probe (1-2 x10^6cpm/ml of hybridization mix), salmon sperm DNA (100 µg / ml of hybridization mix) and plasmid vector DNA without insert (1 µg/ ml of hybridization mix used for quenching the non specific binding of radio labeled probe to the membrane) were denatured by heating for 5 minutes at 100°C and was added to prehybridization mix (20-30 ml/ 10 filters), overnight incubation was done at 42°C with rotation. During the hybridization, the container holding the filters was tightly closed, placed in a plastic bag and sealed to prevent any leakage.

Washing:

After the hybridization was completed, the container was placed straight to drain the liquid down, then with the help of a forceps, membranes were placed in 2x SSC/ 6x SSC buffer facing the DNA downwards, to prevent drying of the filter. Washing was done as follows with high or low stringency wash buffers depending on the choice

High stringency **washing:**

Once with 2 x SSC and 0.1% SDS at room temperature for 10 min.

Once with 2 x SSC and 0.1% SDS at **65°C** for 30 min

Once with 0.2 x SSC and 0.1% SDS at 65°C for 30 min

The last washing step was repeated once or twice increasing the incubation time depending on the background radioactivity on membranes. The radioactivity on the membranes can be upto 100 IPS (impulses per second).

Low stringency washing:

Once with 6 x SSC at room temperature for 10 min

Once with 6 x SSC at 65°C for 30 min

Once with 2 x SSC at 65^0C for 30 min

The last washing step was repeated once or twice increasing the incubation time depending on the background radioactivity on membranes. The radioactivity on the membranes can be upto 100 IPS (impulses per second).

Membranes were placed on the smooth surface of the bench coat paper, the paper was marked with radioactive ink at few points for orientation, wrapped in saran wrap, exposed to X-ray film with intensifying screen. Film was exposed for 12-14 h at -70°C and developed using automatic developing machine.

3.2.20(1 Identification of Positive Plaques and Phage Elution:

After developing the film, it was reoriented on the respective plate according to **the** marks/pricks made both on the plates and membrane filters. Various colonies identified corresponding to the hybridization signal. Once the region was identified, agar plug was taken out with the help of yellow tip (broad end was used) and transferred to 1 ml of SM buffer in sterile eppendorf tube and 20 µl of chloroform was added. Tubes were subjected to vigorous vibrations at 4 C (overnight) for phage elution.

3.2.20e Secondary **and Tertiary Screening:**

Phage titer was determined for the eluted phage as mentioned earlier. For the secondary and tertiary screening care was taken that only 100 pfu were used per plate (10cm plates) and plaques must be well separated. Colony hybridization was done with the same probe as used in the first screening. The phages after the secondary and tertiary screening were eluted in 500 µl of SM buffer.

3.2.20f Amplification of the Phage:

The positive phage clone eluted from third screening was amplified on agar plate. to get a higher titer for large-scale preparation of phage DNA from liquid lysates.

Protocol:

25 ul of the eluted phage (1000-2000 pfu) from 3rd screening diluted to 100 ul with SM buffer and 100 ul of LE 392 cells (A_{600}=2.0) were mixed and incubated at 37^0C for 15 min. Phage infected cells were mixed with 3 ml of 0.7% Top agar (melted, pre cooled to 48°C) and spread uniformly over 10 cm bottom agar plates (minimum 2 days old, pre heated to 37^0C before use). Allowed to cool for 20 min, then incubated in an inverted position at 37^0C in the incubator for 8-10 hours. After appearance of clear plaques, the plates were overlaid with 6 ml of SM buffer and kept for phage elution at 4 C on a flat surface or platform. Eluted phage suspension was transferred to a glass tube (mini culture tubes), plates were rinsed with an additional 2 ml of SM buffer, volume measured and chloroform was added to a final concentration of 5%. The tubes were incubated at room temperature for 15 min with occasional shaking, centrifuged at 4000 x g for 5 min at 4°C. Supernatant was transferred to a fresh sterile glass tube. Chloroform was added to a concentration **of** 0.3% and stored at 4^0C. Titer was determined using the protocol mentioned earlier.

3.2.21 Phage DNA Isolation using QIAGEN Kit:

The titer was determined for the phage amplified after the tertiary screening.

3.2.21a Preparation of Liquid lysate:

Preparation of host cells:

A single colony of LE392 was inoculated into 100 ml LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$, incubated overnight at 30^0C. Centrifuged at 500 x g for 10 min. Supernatant discarded, cell pellet suspended in sterile 10 mM $MgSO_4$. Cells were diluted to a density of A_{600}=2.0 (1.5x 10^9 cells / ml). **1.5x** 10^{10} cells in 10 ml were diluted to 12ml.

Phagc Infection:

To **1.5x** 10^{10} LE392 cells, 5x 10^8 pfu (phage eluted from third screening) were added, incubated at 37^0C for 30 min. The infected cells were added to 400 ml of LB medium prewarmed to 37°C in 1 liter conical flask, incubated at 37°C with vigorous shaking, lysis occurred after 6-7 hours of incubation, prolonged incubation resulted in overgrowth of host cells. 0.4 ml chloroform was added to 400 ml culture and incubated at 37°C with shaking. Cultures were stored at **4°C.** Liquid culture was centrifuged at 10,000 x g for 15 min (JA 10, 5000 rpm), supernatant transferred to a fresh sterile bottle, and centrifugation step repeated to get the clear phage supernatant. Culture (phage) supernatant was prewarmed to 37°C for 15 min.

3.2.21b Lambda Phage DNA Isolation Using QIAGEN kit Protocol:

QIAGEN Protocol:

Before Start:

Dissolve the lyophilized RNase and DNase 1 provided in the kit in buflfer L1.

Prechill buflfer L2 to 4^0C

Check buffer L4 for SDS precipitation due to low storage temperatures, if necessary dissolve SDS by warming.

Phage supernatant stored at 4^0C should be warmed to 37^0C.

Refer to **Table 13** for buffer composition.

Buffer	Composition of the Buffers	Storage
Buffer L1	300 mM NaCl; 100 mM Tris-HCl, pH 7.5; 10 mM EDTA 0.2 mg/ml BSA; 20 mg/ml RNase A, 6 mg/ml DNAse	4 "C
Buffer L2	30% polyethylene glycol (PEG 6000), 3 M NaCl	RT/ 4 °C
Buffer L3	100 mM NaCl; 100 mM Tris-HCl, pH 7.5; 25 mM EDTA	RT
Buffer L4	4% sodium dodecyl sulfate (SDS)	RT
Buffer L5	3 M potassium acetate, pH 5.5	RT
Buffer QBT (Equilib. buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol, 0.15% Triton X-100	RT
Buffer QC (Wash buffer)	**1.0M** NaCl; 50 mM MOPS, pH 7.0; 15% ethanol	RT
Buffer QF (Elution buffer)	1.25 M NaCl: 50 mM Tris-HCl, pH 8.5; 15% ethanol	RT
TE	10 mM Tris-HCl, pH 8.0; 1 mM EDTA	RT

Table 13: The buffer composition of various reagents used for phage DNA isolation

Protocol:

To 250 ml of culture supernatant (taken in 400ml sterile JA10 centrifuge bottle) 400 µl of buffer L1 added and incubated at 37^{0}C for 30 min. 50 ml of ice cold buffer L2 was added to precipitate phage DNA, mixed gently and incubated on ice for 60 min. Centrifuged at 10,000 x g (JA10-5000 rpm) for 10 min and the supernatant was discarded. After centrifugation step and removal of supernatant, the tube was kept upside down for 1 min to allow the residual liquid to drain. (Note: do not wipe out the inside of the tube) The pellet was resuspended in 9 ml of buffer L3 by pipetting up and down over the walls, transferred to a sterile JA 20-50 ml tube. 9 ml of buffer L4

was added mixed immediately by inverting the tube 4-6 times. Incubated at 70°C for 20 min, then chilled on ice. 9 ml of buffer L5 was added mixed immediately and gently by inverting the tube 4-5 times to avoid localized potassium dodecyl sulfate precipitation (the solution turned cloudy and viscous). Centrifuged at 15000 x g (JA 20 rotor 12500 rpm) for 30 min at 4^0C. The supernatant was recentrifuged at 4°C for 10 min at 15000 x g (JA-20 rotor 12500 rpm) to obtain particle free clear lysate. The clear supernatant was applied to QIAGEN tip 500 (Maxi prep) column pre equilibrated with 10 ml of buffer QBT. Column was washed with 30 ml of buffer QC. DNA was eluted with 15 ml of buffer QF.

3.2.21c DNA Precipitation:

To the eluted DNA 0.7 vol. of isopropanol (room temperature) was added, mixed and centrifuged for 30 min at 15,000 x g (JA-20, 15,000 rpm) at room temperature to avoid salt precipitation. Supernatant was removed carefully without disturbing the pellet. DNA pellet was washed with 70% ethanol (to replace isopropanol with more volatile ethanol), centrifuged for 30 min at 15,000 x g (JA-20), supernatant removed and the pellet was air dried for 5 min and dissolved in 100 µl of sterile double distilled water.

3.3 RESULTS

3.3.1 Isolation of Total RNA from Cultured Fish Cells and HepG2 Cells:

The purity and integrity of the total RNA isolated from fish cells (*Xiphophorus*) as well as HepG2 cells was tested by 1% denaturing agarose gel electrophoresis, the total RNA isolated from 40 mg of fish liver tissue, using QIAgen protocol is also shown in the **Figure 23.** Two distinct bands (28s rRNA and 18s rRNA) were seen, 28S rRNA being double the intensity of 18S rRNA The electrophoretic mobility of fish ribosomal RNA was found to be higher compared to human and mouse ribosomal RNA. As there was no literature available indicating the precise size for fish rRNA, a graph was plotted taking the known R_f values of human rRNAs and their corresponding molecular mass in kb. The exact mass of fish ribosomal RNAs was determined from this graph. The values are shown in the **Table 14.**

3.3.2 Degenerate Primers for RT-PCR:

To amplify MPR specific cDNA fragments, the available sequences of chicken and mammalian (human, bovine, rat and mouse) were subjected to multiple sequence alignment and the following degenerate primers corresponding to the highly conserved regions of MPR were selected.

MPR 300:

Sense Primer: MPR 300 sense (**1s**)

Length: 24 bp.

T_m: 70^0C

Position: Position corresponding to the full-length sequence of human MPR 300 is 447-473 bp.

Sequence: 5'TGT **RCA** CTA **YTT TGA TGA** GTG GAG GAC 3'

Figure 23: Purity and Integrity of RNA Preparation. Total RNA was isolated from cultured cells or liver tissue using the RNeasy kit. 5 μg of each RNA preparation was subjected to 1% denaturing agarose gel electrophoresis. **Lane** 1, total RNA from *Xiphophorus* fish embryonic cells; **lane 2,** trout liver total RNA; **lane** 3, human hepatoma cells (HepG2) total RNA.

Table 14: Table showing the calculated molecular mass of the 18 S and 28S rRNA from fish, in comparison to the human and mouse rRNA's.

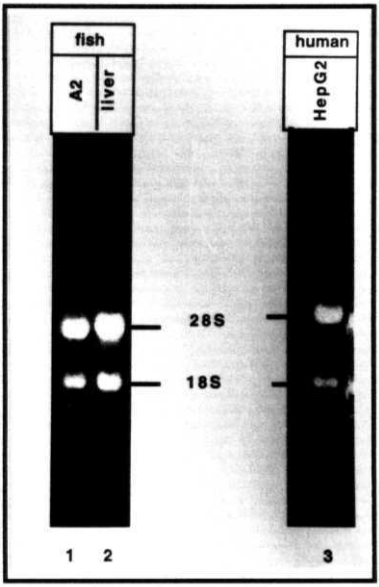


Figure 23

Species	rRNA	Size
Human	18S 28S	1.9 kb 5.0 kb
Mouse	18S 28S	1.9 kb 4.7 kb
Fish	18S 28S	1.8 kb 4.7 kb

Table 14

Antisense Primer: **MPR 300** antisense (2 **as)**

Length: 23 bp.

T_m: 68°C

Position: Position corresponding to the full-length sequence of human MPR 300 is 931-953 bp.

Sequence: 5'CAG GCA TAC TCA GTA AYC CAC TC 3'

MPR 46:

Sense Primer: MPR 46 sense (3s)

Length: 25 bp.

T_m: 70°C

Position: Position corresponding to the full-length sequence of human MPR 46 is 496-520 bp.

Sequence: 5'GAY TGT TTC TAC **CTC TTT** GAG ATG G 3'.

Antisense Primer: MPR 46 antisense **(4as)**

Length: 24 bp.

T_m: 72°C

Position: Position corresponding to the full length sequence of human MPR 46 is 728-750 bp.

Sequence: 5'ATT TCG AGG YTT AGA WCG GCA GAC 3'.

* A/G=R; C/T=Y; A/T=W.

* Expected lengths of the amplified fragments for MPR 46 is 255 bp and for MPR 300 is 506 bp (including the primers).

3.3.3 Amplification of cDNA Fragments Derived from Human MPR's by RT-PCR:

To test the efficiency and specificity of the primers, RT-PCR was performed using total RNA isolated from HepG2 cells as a control. When 10% of RT-PCR product was subjected to 2% agarose gel electrophoresis, cDNA fragments of expected length were seen, an additional band

was seen in case of MPR 46, which could be a non specific band **(Fig 24** A). The DNA bands of expected length were gel purified and sequenced. The nucleotide sequence obtained was compared with the human MPR 300 and MPR 46 sequences and was found to 99% identical, thus confirming the specificity and efficiency of the primers.

3.3.4 Amplification of cDNA Fragments Derived from Fish MPR's by RT-PCR:

RT-PCR was performed with 5 μg of total RNA isolated from *Xiphophorus* embryonic cells for the amplification of fish MPR specific cDNA fragments using **1s** and **2as** primers for MPR 300 and **3s** and **4as** primers for MPR 46 (refer to sequence given under the section 3.3.2).

3.3.4a RT-PCR for MPR 46.

When annealing of primers (3s and 4as) was allowed at 55°C, multiple bands were amplified. The uppermost fragment of about 255 bp **(Figure 24B)** being comparable in length to **the** corresponding human fragment was gel purified and was cloned into a TA cloning vector. The nucleotide sequencing was done using vector derived M13 forward, M13 reverse primers and the primers used for amplification. The nucleotide sequence obtained was translated into protein sequence and was subjected to blast search analysis for sequence comparison. Surprisingly, it was found to be 95% identical to ribosomal protein and there was no homology to MPR 46.

3.3.4b RT-PCR for MPR 300. When the annealing of primers **(1s and 2as) was** allowed at 55°C, no product was amplified.

* For the above PCR reactions normal Taq DNA polymerase (New England Biolabs) was used.

3.3.5 Amplification of cDNA Fragments Derived from Fish by RT, Followed by PCR with Hot Star Taq DNA Polymerase:

Reverse transcription was performed with total RNA isolated from *Xiphophorus* embryonic cells as well as total RNA isolated from trout liver tissue. 5 μg of total RNA was used for first strand synthesis carried out with MPR 300 specific 2as primer. 50% of the RT product was used for the PCR amplification and was performed with Hot Star Taq DNA polymerase kit using MPR 300 specific **1s** and **2as** primers. When annealing was allowed at 45°C, no amplification was seen from fish liver RNA either with or without 'Q' solution (described under methods). In case of fish cell RNA no product was seen in presence of 'Q' solution, but in the absence of 'Q' solution, multiple bands were amplified by RT-PCR, the uppermost fragment of about 0.5 kb being comparable in length to the corresponding human fragment **(Figure 25A).** Raising the annealing temperature above 45°C to increase the specificity of the RT-PCR reaction completely abolished amplification. Therefore, the 0.5 kb fragment obtained with 45^0C annealing temperature was gel purified and reamplified **(Figure 25 B).** The gel- purified fragment was used for TA cloning into pCR 2.1 vector. Positive clones were identified by blue white selection method. 80% of the clones appeared white (could be positive) and the rest were in blue color **(Figure 26).** One of the clones was picked up for plasmid DNA isolation and subjected to restriction analysis with EcoR I **(Figure 27).** The RTP-F1 insert was sequenced from the positive clone using vector derived M13 forward and M13 reverse primers. The nucleotide sequence obtained revealed that a 492 bp fragment **(Figure 28)** which displayed 46-48% similarity at the amino acid level with the corresponding mammalian and chicken MPR 300. Interestingly, from the protein sequence alignment, it was observed that all the cysteines that are involved in disulfide bonding were conserved. The RTP-F1 fragment was used for two purposes, (1) as a probe for northern blotting to look for the mRNA transcript length coding for MPR 300; (2) as a probe for fish cDNA library screening to get the complete coding sequence of fish MPR 300.

Figure 24: RT-PCR for the Amplification of Human MPR Specific cDNA Fragments. **(A)** HepG2 total RNA was used for the first strand synthesis using MPR specific primers (2 **as** for MPR 300 and 4 **as** for MPR 46). For the PCR amplification, two different amounts of first strand synthesis product was used (5 µl in lanes 2,4,6,8 and 15 µl in lanes 3,5,7,9) and was done with MPR 300 specific primers (**1s** and 2 **as)** and MPR 46 specific primers (3s and **4as).** Annealing was done at 55°C. 10% of each RT-PCR product was subjected to 2% agarose gel electrophoresis. **Lane 1,** standard DNA ladder; **lanes 2-5,** amplified cDNA fragments for MPR 46 of expected length of 255 bp in addition to a band of higher mass; **lanes 6-9** amplified cDNA fragments for MPR 300 of expected length of 515 bp.
*For first strand synthesis different amounts of total RNA was used 2 µg in lanes 2,3,6,7 and 5 µg in lanes 4,5,8,9.

(B) RT-PCR for the Amplification of Fish MPR 46 Specific cDNA Fragments. 5 µg of fish total RNA was used for the first strand cDNA synthesis with MPR 46 specific 4as primer. The first strand synthesis product was used for PCR amplification with MPR 46 specific 3s and 4as primers. Annealing was done at 55°C. 10% of RT-PCR product was analyzed by 2% agarose gel electrophoresis. **Lane 1,** RT-PCR product of fish MPR 46, **lane 2,** standard DNA ladder.

Figure 25: Amplification of Fish MPR Specific cDNA Fragments by RT-PCR.
(A) 5 µg of fish total RNA from fish trout liver or *Xiphophorus* embryonal cells A_2 were used as template for first strand synthesis with MPR 300 specific 2 as primer. 50% of RT product was used for each PCR reaction with the MPR 300 specific sense and anti-sense primers (**1s** and 2 **as)** at an annealing temperature of 45^0C with **(lanes** 2 **and** 4) or without **(lanes** 1 **and** 3) 'Q' solution. 10% of each of the PCR product was subjected to 2% agarose gel electrophoresis. **Lanes 1 and 2,** RT-PCR with fish liver RNA; **lanes 3 and 4,** RT-PCR with fish cells RNA; **lane 5,** standard DNA ladder.

(B) 80% of the RT-PCR product (corresponding to the reaction conditions of lane 3 showing multiple bands) was loaded on a 2% preparative agarose gel and the MPR 300 specific ~ 506 bp fragment **(*RTP-F1)** was purified using QIA quick kit. A second PCR amplification was performed under the similar conditions used for first PCR. 10% of the second PCR amplified product was analyzed on 2% agarose gel shown in **lane 6** indicated by an arrow head; **lane 5 and** 7, standard DNA ladder

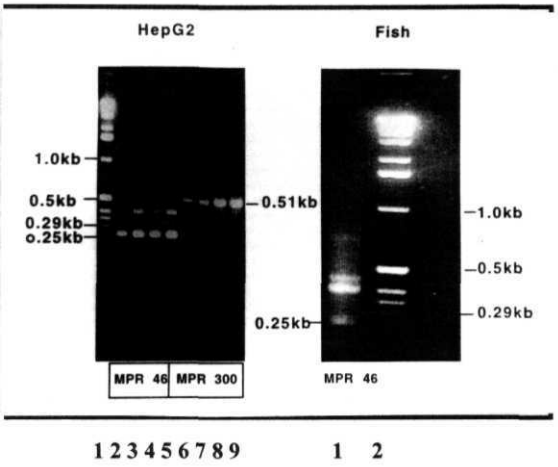


Figure 24

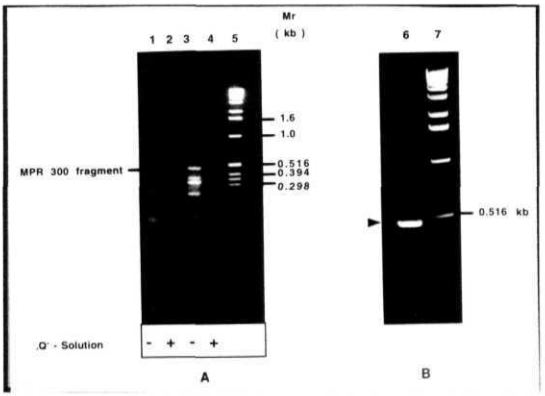


Figure 25

3.3.6 Northern Blotting:

After having confirmed the sequence of RTP-F1 as fish MPR 300 specific cDNA fragment, it was used as a probe for Northern blotting. 15 µg of total RNA isolated from *Xiphophorus* cells was subjected to denaturing agarose (1%) gel electrophoresis (Matzner *et al.,* 1996) and transferred to hybond-N membrane. Membranes were exposed to UV light for 15 seconds and incubated with prehybridization buffer for a minimum of 2 h and then hybridized with ^{32}P labeled **RTP-Fl** fragment. Washing of the membrane was done with high stringency buffer as described under methods. A single band of 13.5 kb was observed **(Figure 29).** This result is compatible with **the** conclusion that the 492 bp long RTP-Fl fragment was derived from fish MPR 300 RNA. mRNA transcript for MPR 300 in mammals and chicken **(Table 15)** have a 7.4-7.5 kb coding region prolonged to 9.5-11 kb by 3' and 5' UTR's (Lobel ***et*** *al.,* 1987; Matzner *et al.*, 1996; Oshima *et al.,* 1988; Szebenyi and Rotwein., 1994).

S.No	Species	**mRNA** Transcript Length for **MPR** 300	Coding Sequence Length
1	Human	~ 9.4 kb	7.473 kb
2	Bovine	~ 9.5 kb	7.497 kb
3	Mouse	~ 9-9.5 kb	7.446 kb
4	Chicken	~ 11.0 kb	7.410 kb
5	Fish	~ 13.5 kb	?

Table 15: Table showing the mRNA transcript lengths coding for MPR 300 **from various species as indicated**

Figure 26: Blue/ White Selection of TA Clones. Fish MPR 300 specific **RTP-F1** fragment (RT-PCR amplified fragment) was cloned into pCR 2.1 TA cloning vector. TOP10F' competent cells were transformed with TA cloned vector and the cells were plated on IPTG/ X-gal coated AMP-agar plates. The positive clones were picked based on blue/white selection. The positive clones appear in white color and (indicated by solid arrow) and negative clones appear in blue color (indicated by dashed arrow).

Figure 27: **Restriction Analysis of Plasmid DNA Isolated from TA Clones.** Plasmid DNA isolated from TA clone was subjected to restriction digestion with EcoR 1 and fragments were analyzed by 2% agarose gel electrophoresis. **Lane 1,** standard DNA ladder; **lane 2,** control plasmid DNA isolated from TA clone; **lane 3,** Plasmid DNA digested with EcoR I.

Figure 29: Northern Blot Analysis. Fish MPR 300 mRNA transcript detected by Northern blot analysis. 15 µg of total RNA isolated from *Xiphophorus* cells was subjected to denaturing 1% agarose gel electrophoresis, transferred to hybond-N nylon membrane and hybridized with ^{32}P labeled fish MPR 300 specific RTP-F1 cDNA fragment. A single band of 13.5 kb mRNA transcript was obtained.

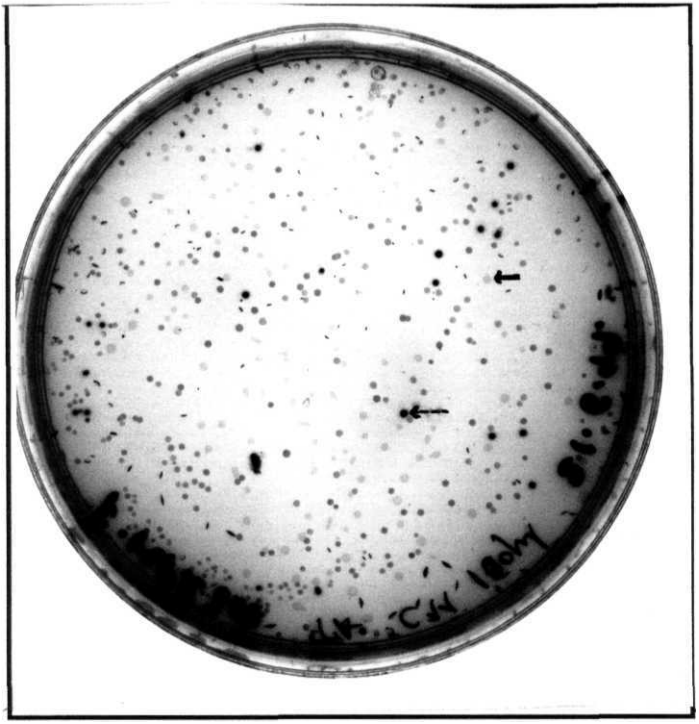


Figure 26

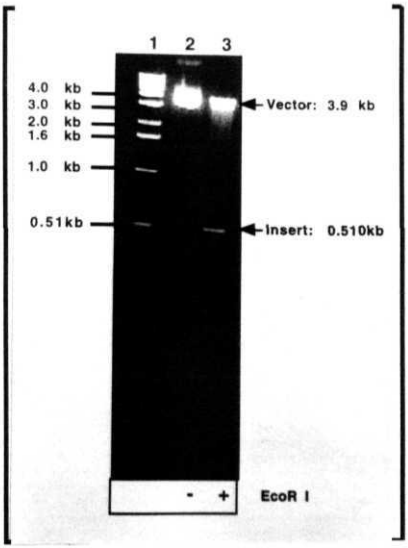


Figure 27

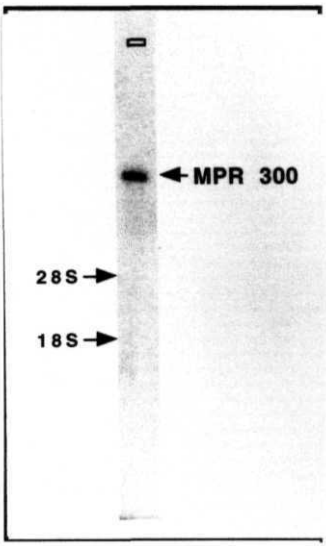


Figure 29

Figure 28: Nucleotide Sequence of RTP-F1 Fragment. The RT-PCR amplified fish cDNA fragment was cloned into TA-cloning pCR 2.1 vector. Sequencing of the insert was done using M13 forward primer. The two degenerate primers specific for MPR 300 used for the RT-PCR amplification is shown by arrows. 1 S and 2 As represent the sense and antisense primers respectively.

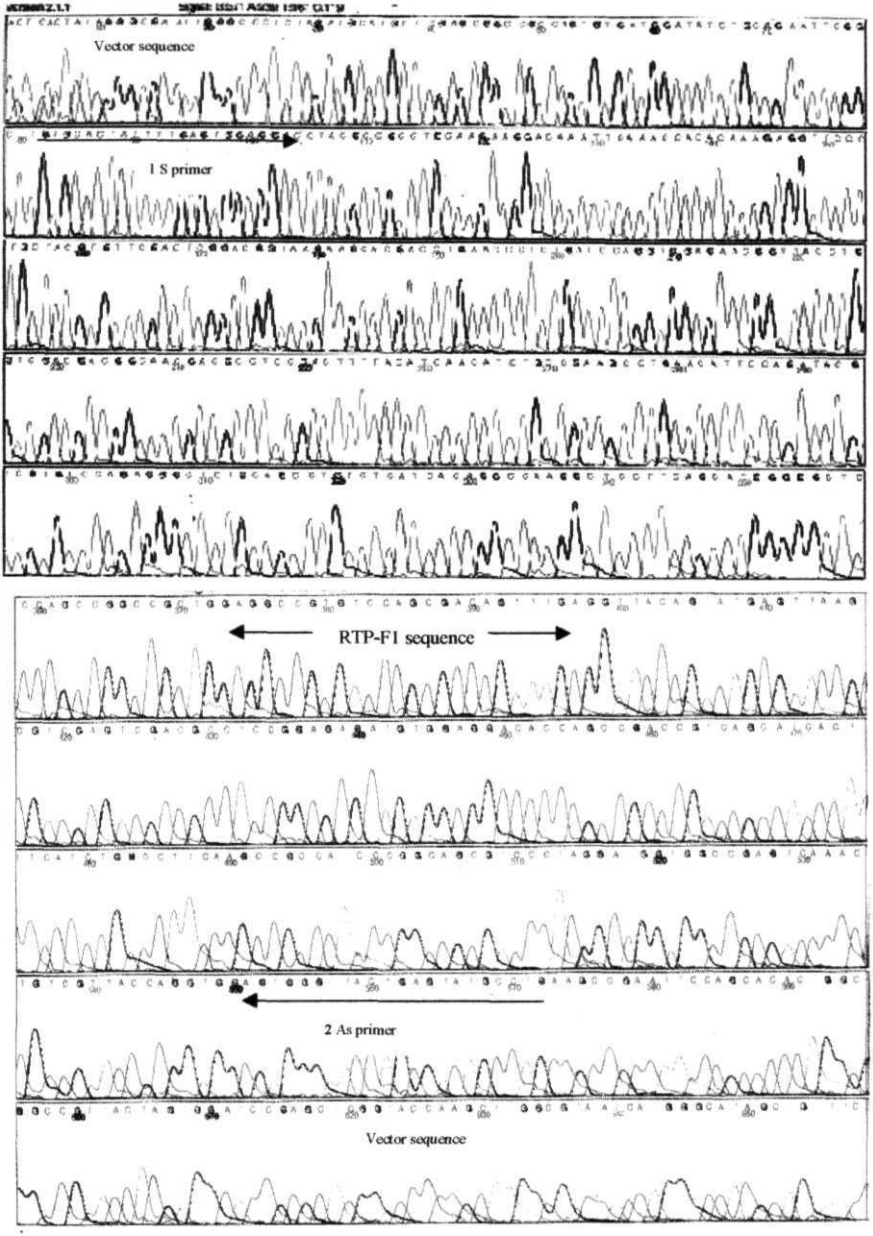

Vector sequence
1 S primer
RTP-F1 sequence
2 As primer
Vector sequence

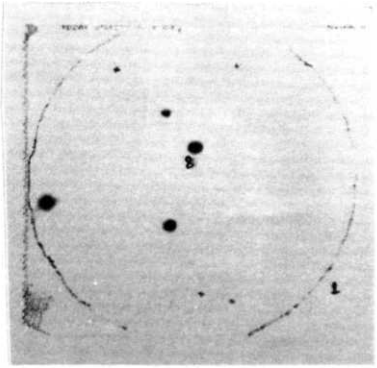
Figure 28

3.3.7 Screening of the Fish cDNA Library Constructed in λZAPII Phage DNA for MPR 300:

The fish cDNA library PSM derived from *Xiphophorus maculatus X. helleri* hybrid fish melanoma (Wakamatsu 1981) and cloned into *X* ZAP II was a kind gift from prof.. Schartl (Wuerzburg, Germany). For the first screening, 10^6 plaque forming units were used to infect E.coli LE392 on 20 plates (14 cm diameter). The primary, secondary and tertiary screenings were done as described under methods using ^{32}P labeled RTP-F1 fragment as a fish derived MPR 300 specific probe. The number of hybridization signals identified in total from all the 20 plates at the primary screening step were 98, 16 were selected randomly from different plates and used for secondary screening. 2 clones were picked from each of the secondary screening plates (in total 5 plates used) and were amplified and verified for the purity by subjecting to tertiary screening. The amplification of the phage clone to homogeneity is shown in **Figure 30.** After the tertiary screening the well-separated positive clones were picked, eluted and used for phage amplification.

Three of the clones (clone 1, 8 and 11) were used for phage DNA isolation, and the phage DNA purified was subjected to restriction digestion with EcoRI to see the insert length. The restriction fragments were resolved by agarose gel electrophoresis. Various clones numbered 1, 8, and 11 were shown to have an insert of ~1.5 kb, ~2.3 kb and ~1.8 kb in length respectively **(Figure 31).** Another restriction analysis was done with various enzymes for clone 8 phage DNA to further confirm the insert size. The fragments were resolved and were transferred to a hybond-N nylon membrane and was probed with ^{32}P labeled RTP-F1 fragment, an insert of 2.3 kb was seen by cutting with EcoRI and EcoR I in combination with Xho **I (Figure 32).** 2.3 kb insert was sub-cloned into pGEM 3Zf(+/-) sequencing vector.

Figure 30: Colony Hybridization and Screening Strategy of Phage Clones (fish cDNA library constructed in *X* ZAP II phage DNA) **with Fish MPR 300 Specific cDNA Probe (RTP-F1).** Among the several clones identified in the primary screening step, only some of them were used for secondary and tertiary screening. As an example one of them (phage clone 8) is shown here which was picked from the primary screening step and subjected to secondary and tertiary screening to get a homogenous preparation and also for the amplification. **Panel A,** primary screening with few hybridization signals, arrow indicates the one which was used for further screening and amplification; **panel B,** secondary screening, **panel** C, Tertiary screening.



A

B

C

Figure 31: Restriction Analysis of Phage Clone DNA. Three different phage clones identified by colony hybridization with ^{32}P labeled fish MPR 300 specific RTP-F1 cDNA fragment, were subjected to secondary and tertiary screening and used for phage DNA isolation. Phage DNA isolated from clones 1,8 and 11 and DNA isolated from the total phage library was subjected to restriction digestion and the fragments were analyzed on 0.7% agarose. **Lane 1,** standard DNA ladder; **lanes 2,4,6 and 9,** Phage DNA digested with EcoR I; **lanes** 3,5,7 **and 10,** phage DNA digested with EcoR I in combination with *Xho* I; **lane 8,** phage DNA isolated from the total library.

Figure 32: Southern Blot Analysis. Phage DNA isolated from clone 8 was subjected to restriction digestion with various enzymes and fragments were resolved by 0.7% agarose gel electrophoresis. DNA was transferred to hybond-N nylon membrane and Southern blot was hybridized with ^{32}P labeled **RTP-F1** fragment to see the exact size of the insert. The bands were detected by autoradiography. **Lane 1,** control clone 8 phage DNA; **lane 2,** Sac **1; lane 3,** Xho 1; **lane 4,** EcoR **I; lane 5,** EcoR I and *Xho* **I; lane 6,** Kpn 1.

* A 2.3 kb insert is clearly seen in lane 4 and 5.

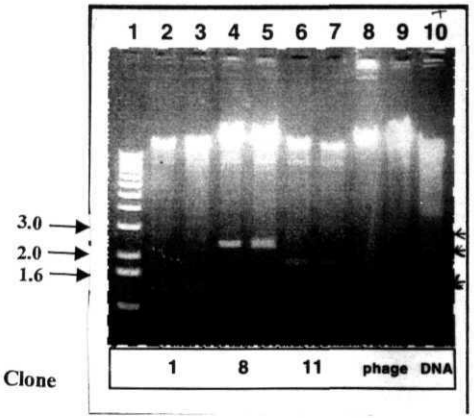
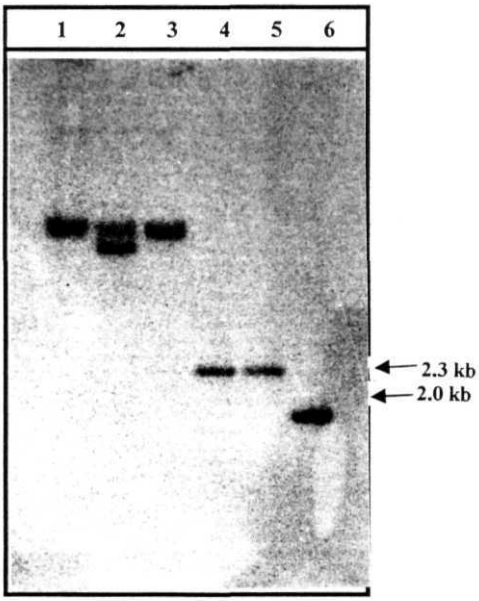


Figure 31



Figure

3.3.8 Sub Cloning of 2.3 kb Insert into pGEM 3Zf (+/-) Vector:

pGEM 3Zf (+/-) vector map is given in the **Figure 33.** 2.3 kb insert cut out from phage clone 8 with EcoRI, both vector and insert were first digested with EcoRI, gel purified and treated with alkaline phosphatase according Sambrook *et al.*, (1989) and were again gel purified. Vector and the insert were mixed in the ratio of 1:3 and were ethanol precipitated, the pellet was dissolved in the required volume of sterile water and ligated. The DH5α competent cells were then transformed with ligated plasmid and transformed cells were plated on Amp-agar plates. Plasmid DNA isolation was done from several clones which were randomly picked. Clones showing exact lengths of vector and insert upon restriction digestion with EcoRI **(Figure** 34) were used for sequencing. Partial sequence was obtained with the vector derived M13 forward, M13 reverse, T7, SP6 primers. Complete nucleotide sequence was obtained with the internal primers designed from the sequence obtained with vector primers **(Table 16).** The nucleotide sequence is shown in **Figure 35.**

The sequence information of ~ 2.3 kb cDNA insert from phage clone 8 was used to amplify a cDNA fragment corresponding to nucleotides 1284-1886 of fish MPR 300 with ULF1 and ULF2 primers **(PCR-F2,** see **Figure 36 B).** PCR-F2 was used a probe to rescreen the *Xiphophorus* library. Phage clones 5 and 7, which were exclusively hybridized with PCR-F2 but not with RTP-F1, were selected for sequence analysis **(Figure 36 B).** Phage clone 5 and 7 inserts were sequenced directly from the phage DNA with M13 forward, M13 reverse universal primers and ULF 7 and ULF8 fish derived primers. The sequence from these clones yielded additional information corresponding to repeat 5 and partial sequence of repeat 6 of fish MPR 300.

Figure 33: pGem 3Zf (+/-) Cloning Vector.

Vector map notes:

1. Sequence reference points:	bp
a.T7 RNA polymerase transcription initiation site	1
b. SP6 RNA polymerase transcription initiation site	69
c. T7 RNA polymerase promoter	3183-6
d.SP6 RNA polymerase promoter	5-61
e. Multiple cloning sites	108
f. *lac* Z start codon	3020-3180; 94-323
g. Binding of M13 forward sequencing primer	3140-3156
h. Binding of M13 reverse sequencing primer	104-120

Figure 34: Cloning of 2.3 kb Fish MPR 300 cDNA Fragment into pGEM 3Zf (+/-) Plasmid Vector and Restriction Analysis. Both insert (2.3 kb cut with EcoR I from phage clone 8) and vector were cut with EcoR I and treated with alkaline phosphatase and were gel purified. Ligation of vector and insert was done (vector to insert ration 1:3). DH5α competent cells were used for transformation. The transformed cells were plated on AMP agar plates. Several clones picked and inoculated into LB medium. Plasmid DNA was isolated from 2 ml of each of the overnight culture and was subjected EcoR I restriction digestion and analyzed by 2% agarose gel electrophoresis. **Lanes 1-4,** negative clones with no insert; **lanes 5-7,** are positive clones with an insert (2.3 kb) and vector (3.199 kb); **lane 8,** is the vector DNA used for ligation; **lane 9,** standard DNA ladder.

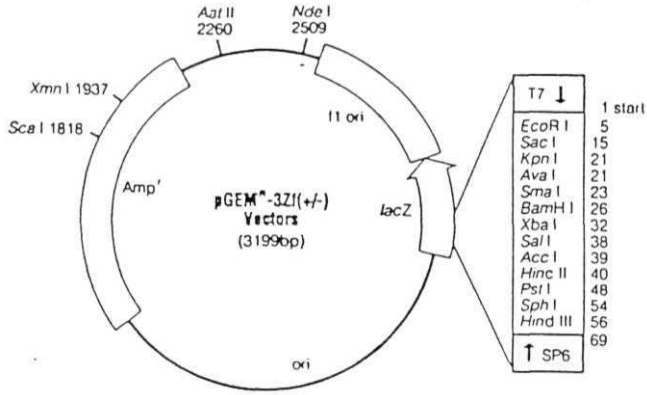


Figure 33

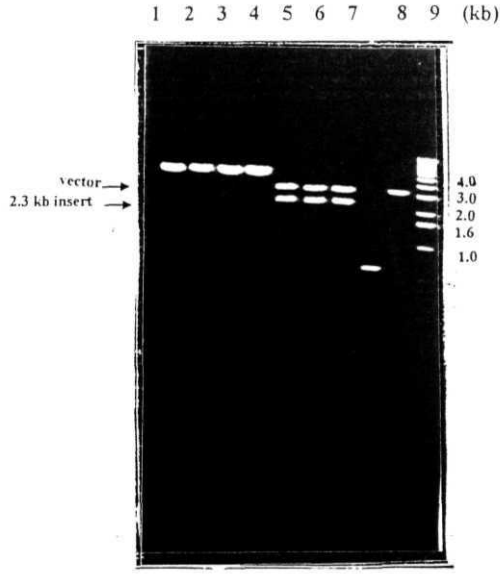


Figure 34

```
          AAT TCG GCA CGA GGA AGG GGA AGA GCC GAG ACA CCA CCT GGG ACA GCT GGC CCA
                    9          18          27          36          45          54

TGG CAA CGA AGG GGC CGA AGG ACA GCG CGA AGA TGG AGA CGA CAA TGC CAC CCA GCG CCA
          63          72          81          90          99         108

GCA GCC GCA ACG GAG CGA AGC TGC TCC ACC TGA TGG AGC CAT CTG GAC GCC AGG TTC TGG
         123         132         141         150         159         168

CTC CCG GGG TTC TAG GGA GCA GAA CCT TCC GGA CCC CCA GCA AAG GAA GGA TAC GGT CGA
         183         192         201         210         219         228

CGA TGA GGA GAC CAA AGT TCC AGA GCA GCA GCA CAG CGA GGA CGA AGG ACG GCC GGC TCA
         243         252         261         270         279         288

GAA CGT CCT GCG GAG CCT TCT GCA CTC GAA TGC ACC GGC AGC ACT CTC TGG CTG CGT AGA
         303         312         321         330         339         348

TGA ACA GCA GGT CGG TGC AGA CCA CCG ACA GCC TCT GGA ACA GTC GGC TCC ACG TTC GGG
         363 ------> 372         381         390         399         408
             3
                       M   F   F   Q   D   K   P   T   Q   R   R   P   L   R
GTC CAG CTG AAT AGA AAC ATG TTT TTC CAG GAT AAA CCG ACC CAA CGC CGG CCT CTG AGG
         423         432         441         450         459         468

L   V   L   W   L   C   I   G   L   G   C   F   L   V   R   S   G   A   E   K
CTG GTT CTG TGG CTG TGC ATC GGT TTG GGA TGT TTT TTG GTT CGG TCC GGA GCA GAA AAG
         483         492         501         510         519         528

V   R   S   S   L   W   Y   Q   D   L   C   S   Y   K   W   E   A   V   D   L
GTC CGC AGC AGC TTG TGG TAC CAG GAC CTC TGC AGC TAT AAA TGG GAG GCT GTA GAC CTG
         543         552         561         570         579         588

D   N   K   V   K   Y   T   I   K   L   C   E   S   S   P   P   T   S   C   G
GAC AAC AAA GTG AAA TAC ACC ATA AAG CTT TGT GAG TCT TCG CCA CCG ACC AGC TGT GGC
         603         612         621         630         639         648

S   S   T   A   V   C   A   Q   S   L   S   G   D   V   K   Y   S   V   G   D
TCC AGC ACT GCA GTT TGC GCC CAA AGC CTC AGC GGT GAT GTC AAG TAC TCG GTC GGT GAC
         663         672         681         690         699         708

L   S   L   Q   K   L   S   G   T   V   L   D   Y  [N   A   T]  D   T   C   P
CTT TCC CTG CAG AAG CTC TCC GGC ACT GTT CTG GAT TAC AAC GCC ACC GAC ACA TGC CCG
         723         732         741         750         759         768

G   G   T   N   P   V   Q   T   S   I   D   F   Q   C   G   K   T   M   G   T
GGA GGT ACC AAC CCG GTT CAG ACC AGC ATC GAC TTC CAG TGT GGG AAA ACC ATG GGC ACC
         783         792         801         810         819         828

P   E   F   V   A   L   S   E   C   V   H   Y   F   E   W   K   T   Y   A   A
CCA GAG TTT GTG GCT CTG TCT GAA TGT GTT CAT TAC TTT GAG TGG AAG ACC TAC GCC GCC
         843         852         861         870         879 ------> 888
                                                             9
C   K   K   D   K   F   K   P   H   K   E   V   P   C   Y   V   F   D   S   D
TGC AAG AAG GAC AAA TTC AAA CCA CAC AAA GAG GTT CCC TGC TAC GTG TTC GAC TCG GAC
         903         912         921 <------                 939         948
                                     5
G   K   K   H   D   L   S   P   L   I   Q   V   E   N   G   Y   L   V   D   D
GGT AAG AAG CAC GAC CTG AGC CCT CTG ATC CAG GTG GAG AAC GGT TAC CTG GTC GAC GAC
         963         972         981         990         999        1008

G   N   D   A   S   D   F   Y   I   N   I   C   R   S   L   N   I   P   D   K
GGC AAC GAC GCC TCC GAC TTT TAC ATC AAC ATC TGC CGA AGC CTG AAC ATT CCA GAT AAG
        1023        1032        1041        1050        1059        1068
```

Continued.

```
S  C  P  E  G  S  A  A  C  L  I  T  G  Q  G  S  F  S  M  G
TCG TGT CCA GAG GGC TCT GCA GCC TGT CTC ATC ACA GGC CAA GGC TCC TTC AGC ATG GGG
      1083       1092       1101       1110       1119       1128

A  P  S  R  P  L  E  A  V  S  S  D  S  L  R  L  Q  Y  E  L
GCT CCC AGC CGG CCG CTG GAG GCC GTG TCC AGC GAC AGT TTG AGG TTA CAG TAT GAG TTA
      1143       1152       1161       1170       1179       1188

S  V  E  S  T  P  P  E  R  C  G  G  H  Q  P  T  V  S  I  T
AGC GTC GAG TCG ACG CCT CCG GAG AGA TGT GGA GGA CAC CAG CCG ACC GTC AGC ATC ACT
      1203       1212       1221       1230       1239       1248

F  I  C  P  S  S  R  H  L  G  S  V  P  R  M  V  A  E  S  N
TTC ATC TGT CCT TCA AGC CGC CAT CTT GGC AGC GTC CCT CGG ATG GTG GCC GAG TCA AAC
      1263       1272       1281       1290       1299       1308

C  R  Y  E  V  E  W  V  T  E  Y  A  C  H  R  D  Y  L  E  S
TGT CGT TAC GAG GTG GAG TGG GTG ACT GAG TAC GCC TGT CAC AGA GAC TAC CTG GAG AGT
      1323       1341       1350       1359       1368
```


```
H  T  C  K  L  T  S  E  Q  H  D  I  S  I  D  L  S  P  L  T
CAC ACC TGC AAA CTG ACG AGC GAA CAG CAT GAC ATC TCC ATC GAC CTT TCA CCT CTC ACC
      1383       1392       1401       1410       1419       1428

Y  G  S  T  E  N  P  Y  F  T  P  S  P  S  G  E  G  S  E  S
TAC GGC TCC ACA GAA AAC CCC TAC TTC ACC CCG TCT CCG TCC GGT GAA GGC TCT GAG AGC
      1443       1452       1461       1470       1479       1488

Y  L  Y  Y  L  N  V  C  G  S  V  S  N  D  L  C  G  N  D  P
TAC CTG TAT TAC CTG AAC GTG TGT GGG AGC GTC TCC AAT GAT CTC TGT GGC AAC GAC CCT
      1503       1512       1521       1530       1539       1548

L  T  S  S  C  Q  V  K  K  S  D  S  T  S  K  V  A  G  R  F
TTG ACC TCA TCC TGC CAG GTC AAG AAG TCC GAT AGC ACC TCT AAA GTG GCC GGA AGA TTC
      1563       1572       1581       1590       1599       1608

Q  N  Q  T  L  R  Y  S  D  G  D  L  S  L  I  Y  P  G  G  D
CAG AAC CAG ACG TTA CGG TAC TCA GAT GGA GAC CTC AGT CTG ATT TAT CCA GGC GGA GAT
      1623       1632       1641       1650       1659       1668

K  C  S  S  G  F  Q  R  M  T  I  I  N  F  Q  C  N  K  T  A
AAA TGT TCC TCT GGC TTC CAG AGG ATG ACC ATC ATC AAC TTC CAA TGC AAC AAA ACC GCA
      1683       1692       1701       1710       1719       1728
```


```
S  N  N  G  H  G  R  P  V  F  A  G  E  T  D  C  T  Y  Y  F
TCA AAC AAC GGA CAC GGC AGA CCG GTT TTT GCC GGG GAG ACG GAC TGT ACG TAT TAC TTT
      1743       1752       1761       1770       1779       1788
```


```
S  W  D  T  A  F  A  C  V  K  E  K  E  D  L  L  C  Q  V  R
AGC TGG GAC ACG GCG TTC GCC TGC GTC AAG GAG AAG GAG GAC CTG CTG TGT CAG GTC AGA
      1803       1812       1821       1830       1839       1848

V  G  S  K  H  Y  D  L  S  R  L  T  R  Y  P  E  S  K  D  G
GTC GGC AGC AAA CAC TAC GAC CTC TCA CGC CTT ACG AGA TAT CCC GAG TCG AAG GAC GGT
      1863       1872       1881       1890       1899       1908

E  N  W  R  V  E  V  G  P  S  A  K  P  D  T  R  Y  F  L  N
GAG AAC TGG AGG GTA GAG GTC GGC CCA TCC GCC AAA CCA GAC ACG CGT TAC TTC CTG AAC
      1923       1932       1941       1950       1959       1968

I  C  H  K  V  L  R  R  G  G  A  A  S  C  P  D  D  A  S  F
ATC TGC CAC AAA GTC CTG AGG AGA GGA GGA GCT GCC AGC TGC CCA GAT GAC GCT TCC TTC
      1983       1992       2001       2010       2019       2028

C  A  V  D  K  N  N  E  T  I  N  L  G  S  F  L  S  P  P  L
TGC GCT GTG GAT AAA AAT AAT GAA ACC ATC AAC CTG GGC AGC TTC CTC TCT CCT CCC CTG
      2043       2052       2061       2070       2079       2088

M  T  K  Q  G  S  D  I  R  L  T  Y  T  E  G  T  T  C  E  N
ATG ACT AAA CAG GGA AGT GAC ATC AGA CTG ACA TAC ACT GAA GGA ACG ACT TGT GAA AAC
      2103       2112       2121       2130       2139       2148
```

Continued.

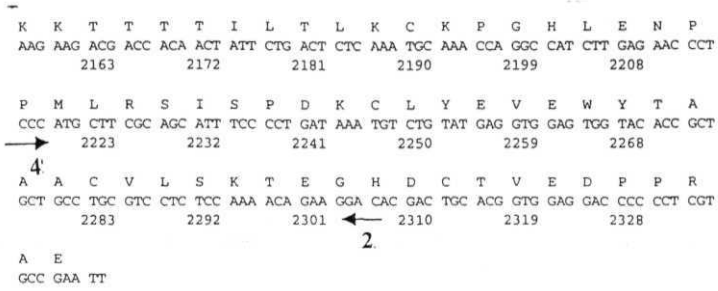


Figure 35

Figure 35: Nucleotide and Deduced Aminoacid Sequence of the 2.34 kb Fish MPR 300 cDNA. 2.34 kb fish MPR 300 cDNA insert isolated from λZAP II phage clone 8 and sub-cloned into pGEM 3zf (+/-) sequencing vector. The insert consists of 432 bp 5' untranslated region followed by coding region corresponding to domain 1-4. The arrow indicate the position of sequencing primer (sense primer (→) and anti-sense primer (←)). The number of each sequencing primer is given below the arrow and the corresponding sequence is given in the **Table 16.** The potential N-linked glycosylation sites are boxed. The conserved motif DLXXL that defines the carboxyl terminal end of each domain is underlined

S.No	Primer (Sense/Antisense)	Primer	Location in bp Corresponding to ORF	Primer Sequence 5' →3'
1	Sense	ULF1	1285 to 1302	CCAATGCAACAAAGCGGC
2	Antisense	ULF2	1870 to 1886	ACCGTGCAGTCGTGTCC
3	Sense	ULF3	-80 to –63 from ATG	AGATGAACAGCAGGTCGG
4	Sense	ULF4	1763 to 1786	CCAGGCCATCTTGAGAACCCTCCC
5	Antisense	ULF5	495 to 513	GAACACGTAGCAGGGAACC
6	Antisense	ULF6	1334 to 1350	CAGTCCGTCTCCCCGGC
7	Sense	ULF7	2367 to 2383	CAACTGGGTGGCGATGG
8	Antisense	ULF8	2367 to 2383	CCATCGCCACCCAGTTG
9	Sense	1s	429 to 452	TGTA/GCACTAC/TTTTGAGTGGAG GAC
10	Antisense	2as	898 to 920	CAGGCATACTCAGTAAC/TCCACTC

Table 16: Various internal sequencing primers (F-stands for fish derived) used for sequencing. 1s and 2as are the degenerate primers used for RT-PCR

3.3.9 Characterization of cDNA and Deduced Amino Acid Sequence of Fish MPR 300:

The sequencing strategy is summarized in **Figure 36.** Overlapping sequences were always identical. The available sequence information comprises a 432 bp 5' untranslated region. The first methionine of the open reading frame (ORF) is surrounded by a nucleotide sequence (AACATGT) which conform with the published consensus sequence for an initiator methionine (Kozak., 1981 and 1986).

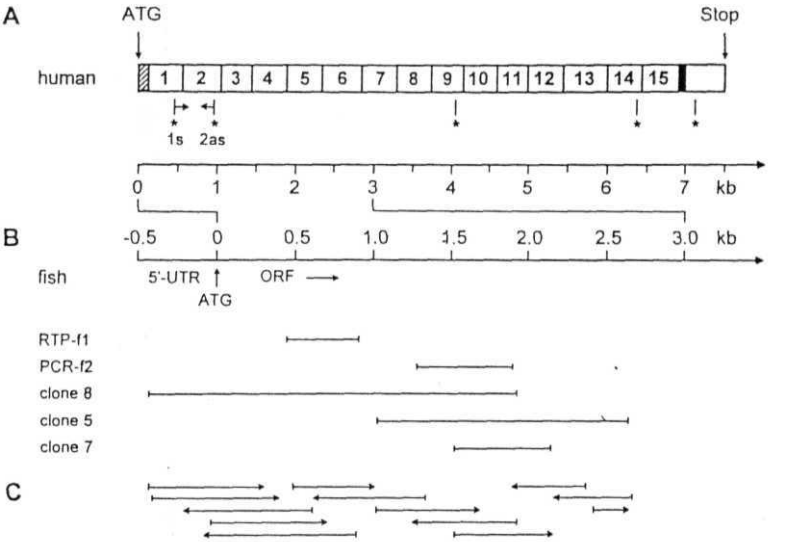


Figure 36

Figure 36: Strategy for cDNA Cloning and Sequencing of Fish MPR 300. A Primary structure of human MPR 300 (Oshima *et al.*,1988). The arrow indicates the initiator methionine (ATG). *Asterisks* indicate stretches of highly conserved nucleotide sequences. 1s, 2as, position of degenerate primers 1 sense and 2 antisense. B cDNA fragments of fish MPR 300 obtained by RT-PCR (RTP-Fl), by phage library screening (clones 5,7,8) and PCR amplification from phage DNA(PCR-F2). C Sequencing strategy. Arrows indicate the length of overlapping sequences

Cassette/Repeat	Chicken	Murine	Rat	Bovine	Human	Function
	Similarity to fish MPR 300 (%)					
1	52	48	49	49	52	
2	52	50	50	48	48	
3	60	55	57	55	56	M6P binding
4	50	51	50	46	48	
5	63	60	62	61	62	
6 (Partial sequence)	52	50	50	48	50	

Table 17: Sequence comparison of internal cassettes in vertebrate MPR 300

The deduced amino acid sequence of the 2638 bp ORF corresponds to cassettes 1-5 and part of cassette 6 of the reported sequences of MPR 300 (cassettes 1-5 are shown in **Figure 37).** Cassette 1 is preceded by a hydrophobic signal sequence. The putative signal peptide cleavage site is between position 32-33: SGA-EK (Nielsen *et ah,* 1997). The carboxy terminal ends of cassettes 1-5 are defined by the conserved motif DLS(P/R/S)L. Cassettes 1-5 of fish MPR 300 show 12-31% similarity with each other and displayed significant sequence similarity with chicken (52%) and mammalian (48-50%) MPR 300 **(Table 17)**

Figure 37

Figure 37: Alignment of the Amino acid Sequence of **Cassettes 1-5 of Fish MPR 300.** From the partial cDNA sequence of fish MPR 300, cassettes 1-5 were selected using the putative signal sequence cleavage site and the conserved motif DLSXL at the carboxyterminal border of each cassette. Cassettes were aligned by the clustalw method. Numbers at the end of each cassette give the carboxyterminal amino acid. Numbers in the brackets give the length of each cassette of fish MPR 300 followed by the range of the length of corresponding cassettes of other vertebrate MPR 300. The bottom lines shows conserved amino acids *(asterisks)* and conservative substitutions *(dots)*. Cysteine residues involved in disulfide bonding are shown in bold, potential glycosylation sites are underlined

3.4 DISCUSSION

MPRs are well characterized in mammals and aves. The luminal domain of MPR 46 displays 14-37% similarity to the 15 internal repeats/ cassettes of MPR 300 as shown for the bovine MPRs (Lobel *et al.,* 1988). This finding led to the assumption that MPR 300 has evolved from MPR 46 by repeated gene duplication events, and raised the question at what stage of evolution MPR 300 has appeared for the first time.

From the previous biochemical and immunological studies, the first evidence was provided for the presence of MPR 300 and MPR 46 in fish (Siva kumar *el al.,* 1999) In order to get further insight into the cDNA sequences of these receptors, various molecular biological methods were used. At first the cDNA sequences encoding mammalian and chicken MPRs were retrieved from the sequence data base. The MPR specific nucleotide sequences were subjected to multiple sequence alignment and searched for highly conserved regions Pair of degenerate primers was selected for both MPR 300 and MPR 46 corresponding to highly conserved regions of MPRs. These primers were used to amplify MPR specific fish derived cDNA fragments.

The fragments of expected length were obtained for both MPR 46 and MPR 300 The nucleotide sequence obtained for the cDNA fragments amplified for MPR 46 was translated into protein sequence and was subjected to blast search analysis for sequence comparison. Surprisingly, it was found to be 95% identical to ribosomal protein and there was no homology to MPR 46 Whereas the nucleotide sequence obtained for the cDNA fragments amplified for MPR 300 revealed that a 492 bp fragment **(Figure 28)** which displayed 46-48% similarity at the amino acid level with the corresponding mammalian and chicken MPR 300. Using the RTP-F1 fragment as a probe, fish cDNA library was screened under high stringency conditions. Several positive signals were seen at the first screening step. These clones were subjected to secondary and tertiary

screening, phage DNA was isolated from some of these clones. One of the clone having longest insert was cut out and sub cloned into pGEM 3Zf (+/-) sequencing vector.

Here we present the partial cDNA sequence of the fish MPR 300. The available sequencing data show that the amino terminal portion of fish MPR 300 displays significant sequence similarity with chicken (52%) and mammalian (48-50%, **Table 17).** The significance of these similarities is corroborated by the fact that fish MPR 300 shares typical structural features with mammalian and chicken MPR 300: First, the amino terminal portion of fish MPR 300 is divided into cassettes which show similar length as their chicken and mammalian counterparts **(Figure 37).** Second. the carboxy terminal border of each cassette is defined by the conserved motif DLS(P/R/S)L which strongly resembles the corresponding sequences found in mammalian and chicken MPRs (DLXXL). Third, all the cysteines which have been suggested to form disulfide bonds in bovine MPR 300 (Lobel *et al.,* 1988) are present in all vertebrate species studied so far, including fish. In most instances, the amino acid sequence flanking a conserved cysteine show remarkably high degree of similarity among all vertebrate species. Fourth, 5 out of 6 potential glycosylation sites found in cassettes 1-5 of fish MPR 300 are conserved in chicken and mammals. Only cassette 4 of mammalian MPR 300 contains two additional glycosylation sites, which are absent both in fish and chicken MPR 300. Taken together, these data suggest that the cDNA sequence presented here encode for fish MPR 300.

Cassettes 3 and 9 of bovine MPR 300 have been shown to contain two independent binding sites for mannose 6-phosphate carrying ligands. Binding of mannose 6-phosphate in both cases depends on a critical arginine residue (R435 in cassette 3 and R1334 in cassette 9, Dahms *et al.,* 1993). Fish MPR 300 contains a corresponding arginine (R422) in cassette 3, which is

surrounded by the highly conserved motif CSSGFQRM (T/S)(I/V)INF(Q/E)C (Bold letters in **Figure** 38 A).

When the sequences of individual cassettes are compared between fish and other vertebrate phyla, similarities are most remarkable for cassettes 3 (55-60%) and 5 (60-63%) **(Figure 38 A** and **B),** whereas cassettes 1,2 and 4 display only 46-52% similarity **(Table 17).** For cassette 3, the high degree of similarity among vertebrates may reflect the presence of critical structural determinants for binding of mannose 6-phosphate containing ligands. For cassette 5, no ligand-binding function has been described yet. We therefore asked whether for the other cassettes of vertebrate MPR 300 the degree of similarity correlates with known ligand binding functions. For this purpose, all cassettes were compared to those of bird (chicken) which, among the phyla studied so far, is closest to fish in the evolutionary tree. The average similarity of cassettes was 57.8% compared to chicken. Remarkably high similarity was observed for cassette 5 (65-69%), and to somewhat lower extent for cassettes 12,14,and 15 (60-64%). Similarity was lowest for cassette 11 (44-47%) which is in line with the observation that chicken MPR 300 does not bind IGF-II, while in mammalian MPR 300 cassette 11 binds IGF-II. Cassette 3 and 9, which contain the mannose 6-phosphate binding site, show average similarity (54-64%). It may be interesting to speculate whether the high degree of conservation of cassettes 5,12,14 and 15 reflects the presence of ligand binding sites, e.g. for retinoic acid (Kang *et al,* 1997), urokinase receptor (Nykjaer *et* ***al,*** 1998), human DNase I (Jerry Cacia., *et al.,* 1998), leukemia inhibitory factor (Blanchard *et al.,* 1999) or other still unknown molecules.

The similarities of individual cassettes of fish MPR 300 to the corresponding cassettes of the other vertebrate phyla is always significantly higher (40-60%) than the similarities among cassettes 1-5 of fish MPR 300 (12-31%). This observation supports the notion that the cassettes

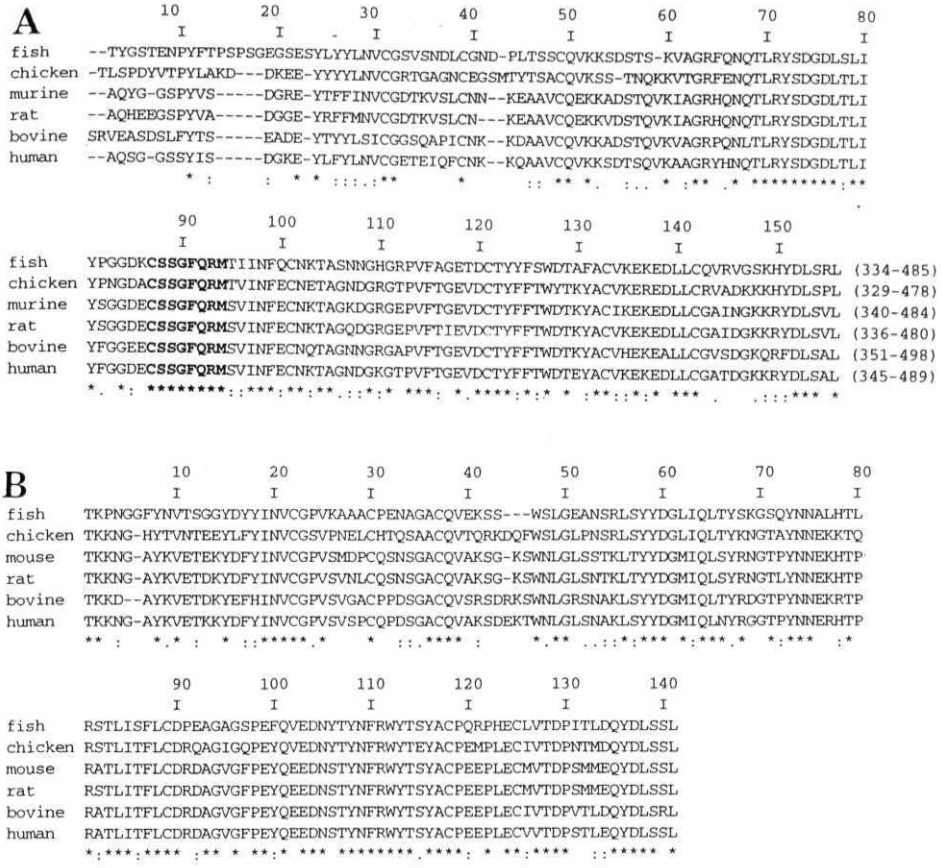


Figure 38

Figure 38: Alignment of Amino acid Sequences of Cassette 3 **and** 5 of Vertebrate **MPR** 300. A. Cassette 3; B. Cassette 5

Cassette 3 and 5 were selected from sequences of fish (present study) chicken (Zhou *et al.*, 1995), mouse (Szebenyi and Rotwein, 1994), rat (Mac Donald, *et al.*, 1988) bovine (Lobel *et a/.,* 1988) and human (Oshima *et al.,* 1988) MPR 300, making use of the conserved motif DLXXL which defines the carboxyterminal end of cassettes. Sequence alignment was performed with clustalw method. The location of cassette 3 in each species within the amino acid sequence of each species is given at the end. The bottom line shows conserved amino acids *(asterisks)* and conservative substitutions (dots). The conserved motif in cassette 3 surrounding the arginine residue which is critical for mannose 6-phosphate binding is shown in bold.

of MPR 300 evolved by gene duplication and diversification long time before fish have been separated from other vertebrate phyla. In this context it is interesting to note that we have recently presented preliminary evidence for the presence of MPR 300 in the invertebrate mollusca, *Unio* (Udaya Lakshmi *et al.*, 1999). The complex extracytoplasmic domain structure of MPR 300 is the most fascinating one to be analyzed for its multifunctional binding properties. These studies clearly indicate that fish MPR 300 show highest sequence homology with chicken MPR 300 when compared to others. Keeping in view of this observation, we intend to use chicken MPR 46 cDNA fragments for screening fish cDNA library at low stringency conditions (Retzek., 1992), this would serve as an alternative approach to fetch fish MPR 46 cDNA. To further understand the evolution of MPRs it will be interesting to investigate the presence and structure of MPR 300 and MPR 46 in other phyla which mark critical points of divergence within the evolutionary tree.

SUMMARY

- The present studies have contributed towards understanding of MPRs in evolution.
- The affinity matrices Sepharose-divinyl sulfone-Phosphomannan (PM gel) and Sepharose-divinyl sulfone-Pentamannosylphosphate (PMP gel) developed by us have potential applications in the purification of both MPR proteins from goat liver on PM gel and their separation on PMP gel.
- Further the PM gel can be used to purify both receptors from the earliest non-mammalian vertebrate fish. The purified receptors have been shown to cross-react with MPR specific antibodies that have been recently shown to immunoprecipitate MPR 300 and MPR 46 from both reptiles and amphibians.
- The molecular mass of fish MPR 46 was found to be 32 kDa under reducing conditions which is lower than that of the receptor from chicken cells (37-40 kDa), whereas the deglycosylated receptor was shown to have similar electrophoretic mobility and molecular mass (26 kDa) to that of the deglycosylated chicken MPR 46, thus representing an underglycosylated form of fish MPR 46. Both chicken and fish MPR 46 bound efficiently to PM gel at pH 6.5.
- Although MPR 46 from chicken and fish are of different molecular mass, they shared a common property of immuno-reactivity with α-MSC1 antibody and efficient binding to PM-gel only when NaCl is completely omitted in the binding buffer.
- MPR 300 protein from fish liver, showed similar molecular mass as other vertebrate MPR 300 proteins and exhibited a pH optimum of 7.0 to bind on PM gel.

- Only MPR 300 was detected and purified from *Unio* (phylum: mollusca) by affinity chromatography employing PM gel. The purified receptor exhibited similar molecular mass as other vertebrate MPR 300 proteins, however the pH optimum for this invertebrate MPR 300 to bind on PM gel was found to be 6.0. Failure to detect MPR 46 in *Unio* could possibly be due to its low expression.
- The presence of MPR 46 in Molluscs can possibly be established by metabolic labeling of snail cell line (*Biomphalariaglabrata*, phylum mollusca) followed by immunoprecipitation and phosphomannan binding as described for chicken and fish MPR proteins as no *Unio* cell line is available.
- The partial cDNA sequence obtained for fish MPR 300 reported here for the first time consists 5' UTR, the initiator ATG and an open reading frame corresponding to N-terminal repeat units 1-5 and part of repeat 6 of mammalian MPR 300.
- The amino acid sequence of fish MPR 300 displayed 48-52% similarity with mammalian and chicken MPR 300. Strikingly, all the cysteine residues involved in disulfide bonding were conserved. The arginine residue in repeat 3, which is critical for the mannose 6-phosphate binding affinity in mammalian MPR 300, was also found in fish MPR 300. The peptide motif CSSGFQRM in the neighbor-hood of this arginine was found to be identical in all mammalian and non-mammalian MPR 300 proteins studied so far.
- In the Northern blot, the size of mRNA transcript encoding for fish MPR 300 was estimated as 13.5 kb.
- Our future endeavors are mainly focussed to get the full-length cDNA sequence of fish MPR 300 and MPR 46, which would unravel the evolutionary conservation of MPRs upto the earliest non-mammalian vertebrate fish studied so far.

partial sequence

```
fish        --------MFFQDKPTQ---RRPLRLVLWLCIGLGCFLVR-SGAEKVRSSLWYQDLCSYK
chicken     ------------MARAA---FAPLLLVLLCLPLGDG-----VAAPVSPDEPFFQDLCSYT
murine      -----MRAVQLGPVPSG--PRVALLPPLLLLLLLA------AAGSAQAQAVDLDALCSYT
rat         -----MRAVLPGPVPPG--PRVALLLPLLLLLLVA------AAGSAQGQAVDLDALCSYT
bovine      MEAAAGRSSHLGPAPAGRPPRCPLLLQLQLLLLLLLLPPGWVPGAAGTQGAEFPELCSYT
human       MGAAAGRSPHLGPAPARRPQRSLLLLQLLLLVA--------APGSTQAQAAPFPELCSYT
                          .       *  *                .    .       ****.

fish        WEAVDLDNKVKYTIKLCESSPPTSCGSSTAVCAQSLSGDVKYSVGDLSLQKLSGTVLDYN
chicken     WEAIDTDKHVLYKINLC--FGVEECGRSSAVCAYDVDKRAYMSVGSLTLREISKTLLVFN
murine      WEAVDSKNNAVYKINVCGNVGISSCGPTSAICMCDLKTENCRSVGDSLLRSSARSLLEFN
rat         WEAVDSKNNAVYKINPCGHVDNPRCGPTSAVCMCDLKSENCRSVGDSLLRSSAKSLLEFN
bovine      WEAVDTKNNMLYKINICGNMGVAQCGPSSAVCMHDLKTDSFHSVGDSLLKTASRSLLEFN
human       WEAVDTKNNVLYKINICGSVDIVQCGPSSAVCMHDLKTRTYHSVGDSVLRSATRSLLEFN
            ***:* .::  *.*: *       ** ::*:*  .:.     ***.  *:  : ::* :*

fish        ATDTCPGG--TNPVQTSIDFQCGKTMGTPEFVALSECVHYFEWKTYAACKKDKFKPHKEV
chicken     TTSKCSQQGSEHRIQSNINFLCGKTLGTPEFVTATECVHYFEWRTFVACKKNLFKPVKEV
murine      TTMGCQPSDSQHRIQTSITFLCGKTLGTPEFVTATDCVHYFEWRTTAACKKDIFKADKEV
rat         TTTGCQPS--EHRIQTSITFLCGKTLGTPEFVTATDCVHYFEWRTTAACKKDIFKADKEV
bovine      TTVNCKQQ--NHKIQSSITFLCGKTLGTPEFVTATDCVHYFEWRTTAACKKNIFKANKEV
human       TTVSCDQQGTNHRVQSSIAFLCGKTLGTPEFVTATECVHYFEWRTTAACKKDIFKANKEV
            :*  *      : :*:.* * ****:******: ::*******:* .****: **. ***

fish        PCYVFDSDGKKHDLSPLIQVENGYLVDDGNDASDFYINICRSLN--IPDK----SCPEGS
chicken     PCYVFDEDLKKHDLSPLIRVPGHYLVDDSD-DDSLFINICRDIG--RSSG-ETMNCPAGS
murine      PCYAFDDKLQKHDLNPLIKLNGGYLVDDSDPDTSLFINVCRDIDSLRDPSTQLRVCPAGT
rat         PCYVFDDKLQKHDLNPLIKLNGGYLVDDSDADASLFINVCRDIDSLRDPSTQLRVCPAGT
bovine      PCYAFDRELKKHDLNPLIKTSGAYLVDDSDPDTSLFINVCRDIEVLRASSPQVRVCPTGA
human       PCYVFDEELRKHDLNPLIKLSGAYLVDDSDPDTSLFINVCRDIDTLRDPGSQLRACPPGT
            ***.** . :****.***:  . *****.:   .::**:**.:                ** *:

fish        AACLITGQGSFSMGAPSRPLEAVSSDSLRLQYELSVESTPPERCGGHQPTVSITFICPSS
chicken     AACLIHEGHAYDVGRPQDQLKRHDKDRLILSYERTYNDEKLNFCLGHNPAVTITFVCPSK
murine      AACLLKGNQAFDVGRPKEGLKLLSKDRLVLTYVKEEG-EKPDFCNGHSPAVTVTFVCPSE
rat         AACLLKGNQAFDVGRPKEGLKLLSKDRLVLTYVKEEG-EKPDFCNGHSPAVTVTFVCPSE
bovine      AACLVRGDRAFDVGRPQEGLKLVSNDRLVLSYVKEGA-GQPDFCDGHSPAVTITFVCPSE
human       AACLVRGHQAFDVGQPRDGLKVVRKDRLVLSYVREEA-GKLDFCDGHSPAVTITFVCPSE
            ****:    ::.:* *   *:   .* * * *          : * **.*:*::**:***.

fish        R-HLGSVPRMVAESNCRYEVEWVTEYACHRDYLESHTCKLTSEQHDISIDLSPLTYGSTE
chicken     RGEESAGPKLTAKTNCRYEVEWVTEYACHRDYLESKSCVLTNEQHDVSIDLSPLTLSPDY
murine      R-REGTIPKLTAKSNCRYEVEWITEYACHRDYLQSESCSLSSEQHDITIDLSPLAQYG-G
rat         R-REGTIPKLTAN--CRYEVEWITEYASHRDYLESETCSLSSEQHDIAIDLSPLAQHEEG
bovine      R-REGTIPKLTAKSNCRFEIEWVTEYACHRDYLESRSCSLSSAQHDVAVDLQPLSRVEAS
human       R-REGTIPKLTAKSNCRYEIEWITEYACHRDYLESKTCSLSGEQQDVSIDLTPLAQSGGS
            * . .: *::.*:  **:*:**:****.*****:*.:* *:. *:*:::** **:

fish        NP--YFTPSPSGEGSESYLYYLNVCGSVSNDLCGND-PLTSSCQVKKS-DSTSKVAGRFQ
chicken     VTP-YLAK----DDKEEYYYYLNVCGRTGAGNCEGSMTYTSACQVKSS-TNQKKVTGRFE
murine      SP--YVS------DGREYTFFINVCGDTKVSLCNN--KEAAVCQEKKADSTQVKIAGRHQ
rat         SP--YVA------DGGEYRFFMNVCGDTKVSLCN---KEAAVCQEKKVDSTQVKIAGRHQ
bovine      DSLFYTS------EADEYTYYLSICGGSQAPICNK--KDAAVCQVKKADSTQVKVAGRPQ
human       S---YIS------DGKEYLFYLNVCGETEIQFCNK--KQAAVCQVKKSDTSQVKAAGRYH
                * :          .* :::.:**       *       :: ** *.   .  * :** .
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fish        NQTLRYSDGDLSLIYPGGDKCSSGFQRMTIINFQCNKTASNNGHGRPVFAGETDCTYYFS
chicken     NQTLRYSDGDLTLIYPNGDACSSGFQRMTVINFECNETAGNDGRGTPVFTGEVDCTYFFT
murine      NQTLRYSDGDLTLIYSGGDECSSGFQRMSVINFECNKTAGKDGRGEPVFTGEVDCTYFFT
rat         NQTLRYSDGDLTLIYSGGDECSSGFQRMSVINFECNKTAGQDGRGEPVFTIEVDCTYFFT
bovine      NLTLRYSDGDLTLIYFGGEECSSGFQRMSVINFECNQTAGNNGRGAPVFTGEVDCTYFFT
human       NQTLRYSDGDLTLIYFGGDECSSGFQRMSVINFECNKTAGNDGKGTPVFTGEVDCTYFFT
            * *********:*** .*: ********::***:**:**.::*:* ***: *.****:*:

fish        WDTAFACVKEKEDLLCQVRVGSKHYDLSRLTRYPESKDGENWRVEVGPSAKPDT-RYFLN
chicken     WYTKYACVKEREDLLCRVADKKKHYDLSPLIRSSESA--QNWEAVDSNLSEEWRKRYYIN
murine      WDTKYACIKEKEDLLCGAINGKKRYDLSVLARHSESE--QNWEAVDGSQAESEK-YFFIN
rat         WDTKYACVKEKEDLLCGAIDGKKRYDLSVLARHSESE--QNWEAVDGSQAESEKRNFFIN
bovine      WDTKYACVHEKEALLCGVSDGKQRFDLSALARHSELE--QNWEAVDGSQREAEKKHFFIN
human       WDTEYACVKEKEDLLCGATDGKKRYDLSALVRHAEPE--QNWEAVDGSQTETKKKHFFIN
            * * :**::*:* *** .   .:::*** * * .*    :**..  .   :     :::*

fish        ICHKVLRRGGAASCPDDASFCAVDKNNETINLGSFLSPPLMTKQGSDIRLTYTEGTTCEN
chicken     VCHKVLKRGGASGCPDAAAICSVDKKNNSKNLGTFVSPPKKVG--ENIQLTYSNGGSCGG
murine      VCHRVLQEGKARNCPEDAAVCAVDK-NGSKNLGKFVSSPTKEK--GHIQLSYTDGDDCGS
rat         VCHRVLQAGKAKNCPEGAAVCAVDK-SGSKNLGKFVSSPTKEK--GHIQLSYSDGDDCGN
bovine      ICHRVLQTGQARGCPEDAAVCAVDK-NGSKNLGRFISSPTREK--GNIQLSYSDGDECGG
human       ICHRVLQEGKARGCPEDAAVCAVDK-NGSKNLGKFISSPMKEK--GNIQLSYSDGDDCGH
            :**:**: * * .**: *:.*:*** . : *** *:*.*       .*:*:*::*  *

fish        KKTTTTILTLKCKPGHLENPPMLRSISPDKCLYEVEWYTAAACVLSKTEGHDCTVEDPQA
chicken     NKKIKTVITLICRPGDLESAPILISEG--VCSFTFDWYTAAACVLSKMEGDNCQVSDPQA
murine      DKKISTNITLVCKPGDLESAPVLRAARSDGCFYEFEWHTAAACVLSKTEGENCTVLDAQA
rat         DKKITTNITFVCKPGDLESAPVLRAAGPDGCSYEFEWHTAAACVLSQTEGENCTVLDAQA
bovine      GQKIITNITLMCKPGDLESAPVLTTSRADGCFYEFEWRTAAACVLSRTEGDNCTVFDSQA
human       GKKIKTNITLVCKPGDLESAPVLRTSGEGGCFYEFEWHTAAACVLSKTEGENCTVFDSQA
             :.  * :*: *:**.**..*:* :      * : .:* ********: **.:* * *.**

fish        GLSFDLSPLTKPNGGFYNVTSGGYDYYINVCGPVKAAACPENAGACQVEKSS---WSLGE
chicken     GFSFDLSPLTKKNG-HYTVNTEEYLFYINVCGSVPNELCHTQSAACQVTQRKDQFWSLGL
murine      GFSFDLSLLTKKNG-AYKVETEKYDFYINVCGPVSMDPCQSNSGACQVAKSG-KSWNLGL
rat         GFSFDLSLLTKKNG-AYKVETDKYDFYINVCGPVSVNLCQSNSGACQVAKSG-KSWNLGL
bovine      GFSFDLTPLTKKD--AYKVETDKYEFHINVCGPVSVGACPPDSGACQVSRSDRKSWNLGR
human       GFSFDLSPLTKKNG-AYKVETKKYDFYINVCGPVSVSPCQPDSGACQVAKSDEKTWNLGL
            *:****: *** :   *.* :  * ::*****.*    *  ::.**** :      *.**

fish        ANSRLSYYDGLIQLTYSKGSQYNNALHTLRSTLISFLCDPEAGAGSPEFQVEDNYTYNFR
chicken     PNSRLSYYDGLIQLTYKNGTAYNNEKKTQRSTLITFLCDRQAGIGQPEYQVEDNYTYNFR
murine      SSTKLTYYDGMIQLSYRNGTPYNNEKHTPRATLITFLCDRDAGVGFPEYQEEDNSTYNFR
rat         SNTKLTYYDGMIQLSYRNGTLYNNEKHTPRSTLITFLCDRDAGVGFPEYQEEDNSTYNFR
bovine      SNAKLSYYDGMIQLTYRDGTPYNNEKRTPRATLITFLCDRDAGVGFPEYQEEDNSTYNFR
human       SNAKLSYYDGMIQLNYRGGTPYNNERHTPRATLITFLCDRDAGVGFPEYQEEDNSTYNFR
            ..::*:****:***.*  *: ***  :* *:***:**** :** * **:* *** *****

fish        WYTSYACPQRPHECLVTDPITLDQYDLSSLSRSTS--ANNWVAMDMTNVSN-QKKYYINV
chicken     WYTEYACPEMPLECIVTDPNTMDQYDLSSLAKSEK-RGENWYAMDNSGPNE-RKKYYINV
murine      WYTSYACPEEPLECMVTDPSMMEQYDLSSLVKSEGGSGGNWYAMENSREHVTRRKYYLNV
rat         WYTSYACPEEPLECMVTDPSMMEQYDLSSLVKFEGGRGGNWYAMENSREHFTRRKYYLNV
bovine      WYTSYACPEEPLECIVTDPVTLDQYDLSRLAKSEGGPGGNWYSLDNGGARSTWRKYYINV
human       WYTSYACPEEPLECVVTDPSTLEQYDLSSLAKSEGGLGGNWYAMDNSGEHVTWRKYYINV
            ***.****: * **:****  ::***** * :     . ** :::        :***:**

fish        CRPIVPVPGCDHDASVCQMKYITEKGSPKEVVSVSSMGKSKKGPIIEARDRLLLEFTDGS
chicken     CRPLLAVPGCDRRASVCQMEYRHDHDSYYEVTSISNLGVASKELVVERLGHILLTYANGS
murine      CRPLNPVPGCDRYASACQMKYENHEGSLAETVSISNLGVAKIGPVVEESGSLLLEYVNGS
rat         CRPLNPVPGCDRYASACQSKYENNEGSLAETVAISNLGVAKTGPVVEESGSLLLEYVNGS
bovine      CRPLNPVPGCDRYASACQMKYQGEQGSYSETVSISNLGVAKTGPMVEDSGSLLLEYVNGS
human       CRPLNPVPGCNRYASACQMKYEKDQGSFTEVASISNLGMAKTGPVVEDSGSLLLEYVNGS
            ***: .****:: **.** :*  ...*  *..::*.:* :.   ::*  . :** :.:**
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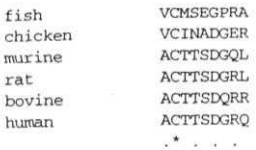

Alignment of Partial Amino acid Sequence of Fish **MPR** 300 **with other Vertebrate MPR** 300 Sequences. Cassette 1 to 5 and partial sequence of 6 were selected from sequences of fish (present study) chicken (Zhou *et al.*, 1995), mouse (Szebenyi and Rotwein, 1994), rat (Mac Donald, *et al.,* 1988) bovine (Lobel *et al,* 1988) and human (Oshima *et al.,* 1988) MPR 300. Sequence alignment was performed with clustalw method The bottom line shows conserved amino acids (*asterisks*) and conservative substitutions (dots)

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Vol. 40, No. 4, November 1996 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
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AFFINITY PURIFICATION OF MANNOSE 6-PHOSPHATE RECEPTOR PROTEINS. PURIFICATION AND PARTIAL CHARACTERISATION OF GOAT LIVER RECEPTORS

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Received August 6, 1996

Summary : Mannose 6-phosphate receptor (MPR) proteins designated as MPR 300 and MPR 46 have earlier been purified from some mammals on phosphomannan coupled to cyanogen bromide activated Sepharose. In a recent study, the goat liver MPR 300 has been directly purified using Sepharose-divinylsulfone-pentamannosyl phosphate matrix (Sivakumar N. 1996, J.Biochem. Biophys. methods, 31, 181-184(1)). In the present report, we describe the preparation of another affinity matrix Sepharose-divinylsulfone-phosphomannan and its utility in purifying the MPR proteins from goat liver. While the MPR 300 from goat liver showed an electrophoretic mobility similar to other mammalian MPRs, the small receptor showed a molecular weight of 36 kDa. Antibodies raised against goat liver MPR 300 react specifically with the large receptor protein. Additionally affinity purified peptide specific antibody corresponding to amino-acid residues 26-42 (ADGCDFVCRSKPRNVPA) of the cytoplasmic tail of the human liver MPR 46 (Pohlmann *et al,* 1988. Proc. Natl. Acad. Sci. USA, 84, 5575-5579 (2)) cross-reacts with the purified small receptor.

Introduction

Lysosomes in cells are the major sites of intracellular digestion and host a large number of hydrolytic enzymes. These are all glycoproteins with a terminal mannose 6-phosphate residue that serves as a recognition marker for the two receptor proteins. In mammalian cells these proteins are known to mediate transport of soluble lysosomal enzymes to lysosomes (3). Although it is unclear as to why the cells express two distinct receptors for the targeting of lysosomal enzymes (4), it is well established that the two receptors bind their ligands in the trans Golgi network (TGN), mediate their sorting from the secretory pathway and deliver

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them to a prelysosomal compartment from where the receptors return to the TGN and the ligands are ferried to dense lysosomes. Recent studies suggest that both the receptors transport distinct complements of lysosomal proteins (5).

The large and small receptors from bovine, human and rat liver are two distinct transmembrane glycoproteins with molecular sizes of 300 kDa and 46 kDa (6). The large receptor has been shown to be structurally and functionally identical to the receptor for insulin like growth factor II (7) and also has a high affinity binding site for IGF-II, although the biological significance of this observation is still unclear. Phosphomannan coupled to Cyanogen bromide activated Sepharose was earlier used for the purification of MPRs from different species (8, 9). Recently the MPR 300 was directly purified from different tissues of rat and goat liver by affinity chromatography employing another matrix Sepharose-divinylsulfone-pentamannosylphosphate (1).

The objective of the present study was to develop a much rapid, inexpensive and convenient affinity method for the purification of both MPRs. We report here the preparation of Sepharose-divinylsulfone-phosphomannan and demonstrate its use for the purification of MPRs from goat liver. The purified proteins have been characterised by biochemical and immunological methods.

Materials and Methods

O-phosphonomannan was a generous gift from Prof. M. E. Slodki (U.S.D.A., Peoria, IL). This was hydrolysed with acid and separated into phosphomannan core (PM) and pentamannosylphosphate (PMP) (1 0). The phosphomannan core was coupled to Sepharose activated by divinylsulfone as described (1), except that in place of pentamannosylphosphate, phosphomannan core was used. In short, 5 ml of Sepharose 6B (Pharmacia) was washed thoroughly with distilled water in a sintered glass funnel and the wet cake was resuspended in 5 ml of carbonate / bicarbonate buffer, pH 11.0. Divinylsulfone (0.5ml, Sigma) was added to this and the suspension gently mixed for 70 minutes at room temperature. The activated gel was washed extensively with water and resuspended in 5 ml of carbonate/bicarbonate buffer, pH 10.0 containing 1 25 mg of phosphomannan core and coupled overnight at 4°C. The coupled gel was washed with water and suspended in carbonate / bicarbonate buffer, pH 8.5 containing 1 25 μl of β-mercaptoethanol and mixed at room temperature for 3 hours. The gel was finally washed with deionised water and suspended in equilibrating buffer : 50 mM imidazole, pH 7.0, 5 mM sodium-β-glycerophosphate, 1 50 mM NaCl, 10 mM $MnCl_2$ and 0.05% Triton X-100 (buffer A).

Extraction of membrane proteins from goat liver : All operations were carried out at 4°C. Fresh goat liver tissue obtained from local slaughter house was cut into small pieces, and the membrane extracts were prepared as described (8). The membrane extracts were resuspended in 50 mM imidazole, pH 7.0, 5mM sodium-β-glycerophosphate, 150 mM NaCl, 1 % Trition X-100 and 0.1 % deoxycholate and stirred overnight at 4°C and the suspension was clarified by centrifugation (4000 rpm, 15 minutes). The clear supernatant was adjusted to 10 mM $MnCl_2$, stirred for 1 hour and the suspension was clarified by centrifugation (9000 rpm, 45 minutes).

Affinity chromatography on Sepharose - divinylsulfone - **phosphomannan gel** : The clear supernatant obtained above was passed through the phosphomannan gel equilibrated with buffer (A) at a flow rate of 20 ml/hr in cold. The gel was extensively washed with buffer (A) and eluted with 5 mM mannose 6-phosphate in the same buffer.

SDS-PAGE : This was carried out on 10% poly acrylamide gels according to Laemmli (11) under reducing conditions, and proteins were detected by silver staining.

Immunological studies : *Raising antibodies to the purified goat liver large receptor* /The large mannose 6-phosphate receptor protein from goat liver was specifically isolated using the affinity matrix Sepharose-divinylsulfone-pentamannosylphosphate (1). Antibodies to the affinity purified goat liver mannose 6-phosphate receptor (MPR 300) were raised in rabbit. 250 μg of the protein in 0.5 ml was emulsified with 500 μl of Freund's complete adjuvant and injected subcutaneously into the rabbit. Rabbit received a booster dose in the third and fifth week (incomplete adjuvant). Rabbit was bled 10 days after the third injection and the blood was allowed to clot, serum separated by centrifugation and stored at -20°C in aliquots. *Raising antibodies to the synthetic peptide and purifying peptide-specific antibodies :* The synthetic peptide corresponding to residues 26-42 of the cytoplasmic tail of human liver MPR 46 (numbering of the tail starts with Arg-21 2 (2,12)) was synthesized using F-moc-protected PyBOP. (benzotriazol-l-yl-oxy-tris-pyrrolidinophosphonium hexaflurophosphate) activated amino acids and an automatic peptide synthesizer (Milliegen 9050, Eschborn, Germany). The peptide was purified by reverse-phase chromatography and was kindly provided by Prof. K. von Figura's group, (Goettingen, Germany). The purified peptide was conjugated to keyhole limpet hemocyanin using gluteraldehyde as described (12). The conjugate corresponding to 250 μg peptide was mixed with Freund's complete adjuvant (first injection) or incomplete adjuvant (booster injections) and subcutaneously injected into rabbits. Booster injections were given 4 weeks after the first injection in biweekiy intervals. For the purification of peptide-specific antibodies, 7 mg of the synthetic peptide was coupled to 1 ml of Affi-gel 10 (Bio-Rad, Munich, Germany) according to the manufacturers instructions. Affinity purificaion of Antibodies was performed as described. (13)

Western blot analysis : The immunoreactivity of the large and small receptors from goat liver with the antibodies raised against large receptor and the antibodies against synthetic peptide of the human liver MPR-46 was detected by Western blot analysis which was performed according to the protocol supplied by Promega (14). The two mannose 6-phosphate receptors from goat liver were separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions. Then the antigens were electrophoretically transferred from the gel to nitrocellulose sheet, (30V, overnight). The nitrocellulose paper was blocked to prevent **non** specific binding and probed with the respective primary antibodies of appropriate dilutions. The Ag-Ab complex was detected by secondary antibody conjugated to alkaline phosphatase and visualised by incubating the membrane with the substrate 5-bromo, 4-chloro, 3-indolyl phosphate/Nitro blue tetrazolium (Bangalore Genei, India).

Results

Mannose 6-phosphate receptor proteins from the membrane extracts of goat liver were isolated by their ability to bind speicifically to the Sepharose-divinylsulfone-phosphomannan gel (PM gel) prepared. After passing the Triton extract through the affinity matrix, two proteins could be eluted specficially using 5 mM mannose 6-phosphate in buffer (A). No protein could be eluted when 10 mM glucose 6-phosphate was used. The eluted fractions were pooled, dialyzed, concentrated and subjected to SDS-PAGE analysis. Figure 1 shows the two receptor proteins with apparent molecular sizes of 300 kDa and 36 kDa respectively. Thus the gel matrix prepared (10mg phosphomannan coupled per ml of gel determined as mannose equivalents) is useful to purify both the large and small receptors from goat liver (19). About 1 mg of purified large and small receptor protein was obtained (estimated by BCA method (15)) from 100 gms of fresh goat liver tissue.

The homogeneity of the receptors was further confirmed by their specific reaction to the antibodies raised against the goat liver large receptor and affinity purified antibody raised against a synthetic peptide in the cytoplasmic tail of the human liver small receptor. Figure 2A showsthe immunoreactivity of goat liver large receptor with the antiserum raised against the purified large receptor (1). Figure 2B shows the immunoreactivity of the goat liver small receptor with an affinity purified peptide-specific antibody (0.45 mg of immunoglobulin obtained from 1 ml of antiserum) using peptide-affinity column.

Discussion

In the present study the large and small mannose 6-phosphate receptor proteins were purified from goat liver in a single step for the first time by affinity chromatography on Sepharose-divinylsulfone phosphomannan gel matrix. The same matrix was found useful to purify the fish liver MPR 300 (Siva Kumar, *et al* under preparation). The receptor proteins from bovine and human liver have previously been purified on phosphomannan coupled to cyanogen bromide activated Sepharose (9). The new affinity matrix described here offers

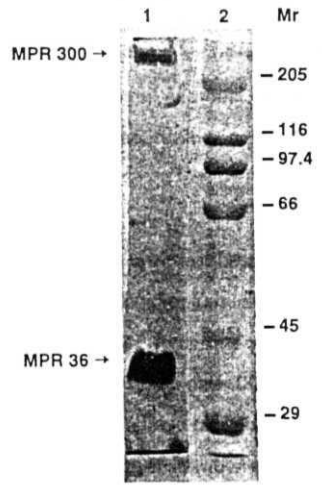

Fig. 1 : SDS-PAGE of affinity purified goat liver MPRs. Arrow indicates position of MPR 300 and MPR 36 respectively. Lane 1 : Purified large and small receptors. Lane 2 : Standard molecular weight markers (Sigma).

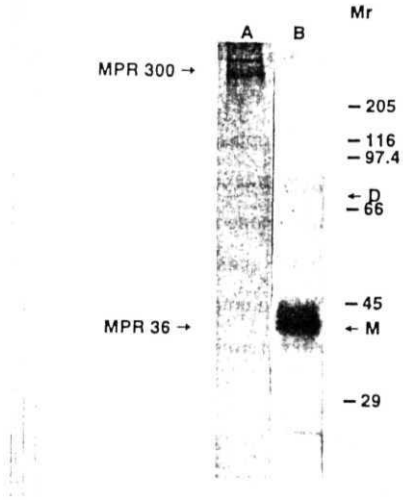

Fig. 2 : Immunoreactivity of goat liver MPRs. A : MPR-300, B : MPR-36. M-Monomer, D-Dimer.

the advantage that activation of Sepharose can be done at room temperature and coupling can be completed by an overnight incubation of the phosphomannan core to the activated gel. The author's laboratory has successfully coupled several carbohydrates such as mannose, N-acetyl glucosamine, lactose and pentamannosylphosphate to Sepharose derivatized by divinylsulfone, and these matrices have been used to purify lectins from a wide variety of sources (1, 16-18).

The fact that the two proteins eluted from the PM-gel are the putative MPR 300 and MPR 46 is not only confirmed by their abilities to bind on PM gels but also by their electrophoretic mobilities as well as by their immunoreactivity. MPR 300 from goat liver, apart from reacting with antibody to goat liver MPR 300, also cross reacts with an antibody to rat liver MPR 300 (data not shown), suggesting antigenic similarities among the different mammalian MPR 300. Although the small receptor from different sources described in literature exhibit a molecular weight of 46 kDa, the goat liver small receptor migrated with an apparent molecular mass of 36 kDa under reducing conditions. In spite of this difference in molecular size as compared to other mammalian receptors, the goat liver receptor cross-reacts specifically with an affinity purified antibody raised against the synthetic peptide described. This antibody also cross reacts with the small MPR from rabbit liver (Udaya Lakshmi Y. & Siva Kumar N. unpublished observation). It has also been observed that the goat liver MPR cross-reacts with an affinity purified cytoplasmic tail specific antibody to the human MPR 46 (Siva Kumar *et al* unpublished observation). Taken together, these data suggest that the cytoplasmic tail of the small receptor among different mammalian species is antigenically similar. From these data it is tempting to speculate that the difference in molecular size of goat receptor as compared to other mammalian receptors (5,8) is possibly due to the difference in the structure of the amino-terminal domain.

In summary, the PM gel developed in this study seems to have potential application to purify MPR proteins from different animal species. Studies are in progress to determine the ability of goat MPR 300 to bind Insulin like growth factor - II, and also to identify the ligand binding sites in the MPR proteins.

Acknowledgements : This work was partially supported by a research grant from the Department of Science and Technology, Government of India. (SR/0Y/GB-24/93) to SKN. YUL acknowledges receipt of fellowship from Council of Scientific and Industrial Research. (India). Part of this work was reported in the M.Phil dissertation of Y. Udaya Lakshmi (1 9).

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Comparative Biochemistry and Physiology Part B 00 (1999) 1–5

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Mannose 6-phosphate receptors (MPR 300 and MPR 46) from a teleostean fish (trout)

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Abstract

Mannose 6-phosphate receptors (MPRs) are known to occur in mammals, birds, reptiles and amphibians. Here we provide evidence for the presence of two MPRs in fish, the earliest vertebrates. Using phosphomannan-Sepharose affinity chromatography, MPR 300 was purified from liver membrane extract of trout. The purified trout liver MPR 300 showed similar electrophoretic mobility as the goat liver receptor and a pH optimum of 7.0 for binding to phosphomannan. The presence of MPR 46 in fish was shown by metabolically labelling embryonic fish cells ***(Xiphophorus)*** and immunoprecipitation with an antibody against the cytoplasmic tail of human MPR 46 (anti-MSCI). This antibody had recently been shown to iminunoprecipitate MPR 46 also from reptiles and amphibians. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Affinity chromatography; Mannose 6-phosphate receptor; Non-mammalian vertebrates; *O*-phosphonomannan; Sepharose divinylsulfone phosphomannan; Teleostean fish; Trout liver; *Xiphophorus*

1. Introduction

Mannose 6-phosphate receptor proteins, designated as MPR 300 and MPR 46, are type 1 integral membrane glycoproteins that recognise and bind to mannose 6-phosphate-containing lysosomal enzymes, and mediate the transport of these enzymes to a prelysosomal compartment. In humans, absence of this recognition system leads to severe lysosomal storage disease emphasizing an essential role in the biogenesis of lysosomes [22]. The bovine and human receptors purified on phosphomannan Sepharose matrix are the most extensively characterized MPRs [9,20], for review see Ref. [8]. The expression levels of these receptors in different tissues have been found to vary [24]. It has also been shown that both receptors transport distinct complements of lysosomal enzymes [15,16]. Among the two receptors, only the mammalian MPR 300 has so far been shown to be a multifunctional protein containing a high affinity binding site for IGF-II and a binding site for retinoic acid [7,11,17]. Our long term objectives are to gain an understanding of the evolution of these receptors. Our previous studies established the presence of both MPR proteins **in** reptiles and amphibians [18]. The objective of the present study was to gain further insight into the evolution of these receptors and to identify these proteins in fish as the earliest non-mammalian vertebrates. We provide here the first biochemical and immunological evidence for the occurrence of both MPRs in fish.

2. Materials and methods

2.1. Materials

O-phosphonomannan (a generous gift of Dr M.E. Sledki, USDA, Peoria, IL, USA) was hydrolyzed and

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PII: S0305-0491(99)00061-9

separated into phosphomannan core (PM core) and pentamannosyl phosphate (PMP) as described [3]. Fresh liver tissue of trout (*Salmo gairdneri* cf.) was obtained locally in Gottingen. An embryonic cell line of *Xiphophorus xiphidium* A-2 [12] was kindly provided by Professor Schartl, Institut für Physiologische Chemie, Würzburg, Germany. Divinyl sulfone, glucose 6-phosphate and mannose 6-phosphate were from Sigma (St. Louis, MO, USA). Rabbit antibodies against the cytoplasmic domain of human MPR 46 (anti-MSCl antibody) and goat liver MPR 300 were prepared as described elsewhere [18]. All other reagents and chemicals used were of high purity and were purchased locally.

2.2. Extraction of membrane proteins from fish liver and affinity chromatography of the membrane extracts on phosphomannan-Sepharose

Membrane proteins from fish liver were extracted following the protocol described [21]. 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 extracts on phosphomannan-Sepharose (PM-Sepharose) was carried out as described previously [18]. The mannose 6-phosphate eluates were concentrated by ultrafiltration (Amicon PM 10) and aliqouts of the concentrates were subjected to SDS-PAGE analysis.

2.3. Iodination of purified MPR 300 and rechromatography on PM-Sepharose

The mannose 6-phosphate eluates concentrated as above were acetone-precipitated and iodinatcd as described [23]. Iodinated receptors were purified on PM-Sepharose (0.2 ml) equilibrated with buffer A (50 mM imidazole-HCl pH 7.0, 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05% Triton X-100, 10 mM manganese chloride). Columns were washed with buffer A and eluted sequentially with three volumes each of 5 mM glucose 6-phosphate followed by 5 mM mannose 6-phosphate in buffer A. Radioactivity was measured in a gamma ray counter, and the fractions were precipitated with trichloroacetic acid and then analysed by SDS-PAGE.

2.4. pH optimum of binding to PM-sepharose

Purified iodinated receptor was diluted in binding buffer (modified buffer A with 50 mM imidazole pH 6.0-7.5 or with 50 mM sodium acetate pH 4.0-5.5 instead of imidazole) to 200 µl and passed through PM-Sepharose (0.2 ml) equilibrated with the corresponding buffer. The columns were washed and sequentially eluted with 5 mM glucose 6-phosphate followed by 5 mM mannose 6-phosphate in the same buffer. Trichloroacetic acid precipitable radioactivity in the column fractions was monitored in a gamma ray counter.

2.5. Metabolic labelling of Xiphophorus cells, binding to PM-sepharose and immunoprecipitation of MPR 46

Xiphophorus cells were cultured in Hams F12 containing 10% fetal calf serum at 28°C in 95% air + 5% CO_2. Cells were grown in monolayers on 6cm plates, incubated in methionine-and cysteine-free medium for 1 h, and 400 µCi of (^{35}S) methionine and cysteine mixture was added. Labelling was done for 8 h. Chicken embryonic fibroblasts known to contain both MPR proteins [15] were used as control cells. After labelling, cells were scraped from the plates with a rubber policeman, and the labelled cell pellets were processed for extraction of the membrane proteins with 50 mM imidazole buffer pH 7.0 containing 0.5% Triton X-100, 1 mM EDTA, 5 mM iodoacetic acid and 1 mM PMSF. To the labelled membrane extract 10 mM each of $MnCl_2$, $MgCl_2$, $CaCl_2$ were added, incubated on ice for 15 min and centrifuged at 109 000 x *g* for 20 min. The clear supernatant was then applied to PM-Sepharose (0.2 ml) equilibrated with column buffer (50 mM imidazole buffer pH 7.0, 5 mM sodium β-glycerophosphate, 0.5 mg/ml BSA, and 10 mM each of $MnCl_2$, $MgCl_2$ and $CaCl_2$). The column was washed extensively with wash buffer (0.05% Triton X-100 in column buffer) and then sequentially eluted with 5 mM glucose 6-phosphate followed by 5 mM mannose 6-phosphatc in wash buffer. Unbound material as well as the eluates were subjected to immunoprecipitation using anti-MSCl antibody as described [15], and MPR 46 was resolved by SDS-PAGE and detected by fluorography.

3. Analytical methods

Protein concentration was estimated with bicinchoninic acid (Sigma). SDS-PAGE was performed according to Laemmli [13]. Where indicated, proteins were reduced by 10 mM DTT for 5 min at 95°C and alkylated with 50 mM iodoacetic acid for 15 min at 37°C. When radioactive samples were used, gels were processed for fluorography [2]. Silver staining of proteins in polyacrylamide gels was performed as described [I]. Immunoprecipitated chicken and fish MPR 46 were subjected to deglycosylation following the protocol described [19].

4. Results and discussion

It has been established that both MPR proteins are present in reptiles and amphibians [18]. In the present report, we investigated the presence of both receptors in fish which are the earliest vertebrates. For the purification of mannose 6-phosphate receptors from fish liver, a detergent extract of the tissue was subjected to affinity chromatography on PM-Sepharose. When the mannose 6-phosphate eluates from PM-Sepharose affinity chromatography were analysed on SDS-PAGE and silver staining, a band with an apparent molecular mass of 300 kDa, the typical electrophoretic mobility of mammalian MPR 300 was detected (data not shpwn). From 200 g of fresh liver tissue about 10-15 μg of protein was obtained. MPR 46 was not detectable in the mannose 6-phosphate eluate. The available data do not allow to decide whether the concentration of MPR 46 is too low in fish liver 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. 1). Under reducing conditions, the electrophoretic mobility of MPR 300 from both species decreased to the same extent (Fig. 1). It is interesting to note that the receptor from the non-mammalian vertebrates chicken [4,5], reptiles and amphibians [18] and fish (present study) show similar molecular mass and electrophoretic behavior. When radioiodinated fish liver MPR 300 protein was reapplied to PM-Sepharose 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. 2). These results confirm that the MPR 300 proteins from the liver tissues of all non-mammalian vertebrate species tested (reptiles, amphibians and fish) show similar binding to phosphomannan as the mammalian MPR 300 protein. Binding was stable to high ionic strengths (0.5 M NaCl, data not shown). The optimal pH for binding to phosphomannan was 7.0 with lower efficiency of binding within the range of pH 6.0-pH 7.5 (Fig. 3). As described earlier for **mammalian** MPR 300 [10], essentially no binding was observed at pH 5.5 or below.

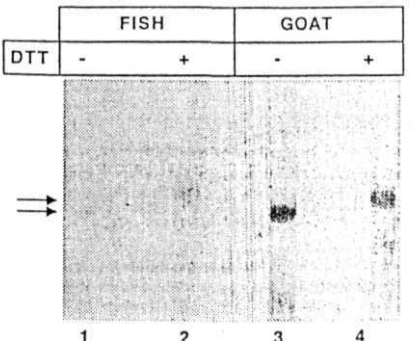

Fig. 1. Apparent molecular mass of radioiodinated MPR 300 from fish liver (lanes 1 and 2) and goat liver (lanes 3 and 4) on 6% polyacrylamide gels under non-reducing (lanes 1 and 3) and reducing conditions (lanes 2 and 4).

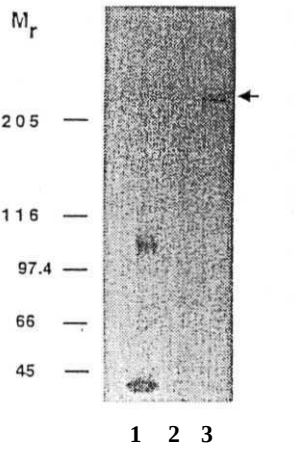

Fig. 2. Affinity chromatography of radioiodinated fish liver MPR 300 on immobilised phosphomannan at pH 7.0. Column fractions were analysed by SDS-PAGE (6% gel) and subjected to fluorography. Lane (1) Flow through; (2) 5 mM glucose 6-phosphate eluate and (3) 5 mM mannose 6-phosphate eluate. Arrow shows position of MPR 300.

The presence of MPR 46 in fish was investigated by nietabolically labelling fish cells and binding the MPR 46 protein to PM-Sepharose and eluting with 5 mM mannose 6-phosphate, followed by immunoprecipitation with the tail specific antibody (anti-MSC1), which has been shown to react with non-mammalian MPR 46 [18]. Preliminary experiments had shown that MPR 46 from non-mammalian cell lines (chicken and fish) did not bind to phosphomannan under the buffer conditions used earlier for mammalian receptors (buffer containing 150 mM NaCl) [20]. In contrast, MPR 300 from chicken has efficiently bound under these conditions (data not shown). Addition of 10 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ to a modified column buffer with NaCI reduced to 90 mM to compensate for the increase in osmolarity, did not improve binding of MPR 46 from chicken or fish. Only when NaCI was omitted,

MPR 46 from chicken and fish cells efficiently bound in the presence of divalent cations and specifically eluted with 5 mM mannose 6-phosphate, but not with glucose 6-phosphate (shown for the fish receptor in Fig. 4, lanes 1-3).

The electrophoretic mobility of the fish protein eluted from PM-Sepharose (32 kDa under reducing conditions) is lower than that of the receptor from chicken cells (37-40 kDa). Three lines of evidence support the assumption that this fish protein is indeed related to MPR 46 from other vertebrates: First, the 32 kDa protein in bound and unbound fractions of PM-Sepharose cross-reacts with the affinity-purified anti-MSC1 antibody raised against the conseryed cytoplasmic domain of mammalian MPR 46. Second, the reduced and alkylated fish receptor (Fig. 4, lane 3) shows a decreased electrophoretic mobility compared to the non-reduced (Fig. 4, lane 4), which indicates that it contains internal disulphide bonds like the mammalian and avian MPR 46 [8,15]. Third, when the fish receptor was deglycosylated by PNGase, its electrophoretic mobility (26 kDa) was similar to that of deglycosylated chicken MPR 46 (Fig. 4, compare lanes 6 and 7). Taken together, our data suggest 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.

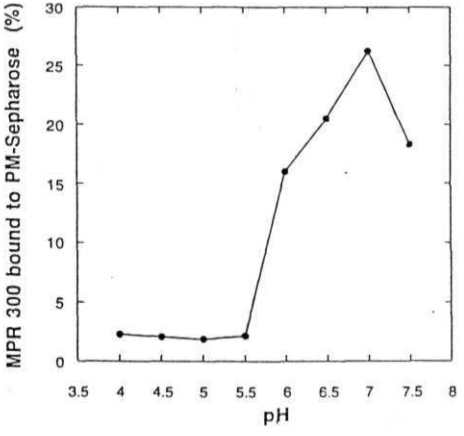

Fig. 3. Effect of pH on the binding of fish liver MPR 300 to immobilised phosphomannan. Radiiodinated MPR 300 was bound to 0.2 ml of PM-Sepharose. After extensive washing, columns were eluted with 5 mM mannose 6-phosphate. Flow through and hound material was subjected to trichloroacetic acid precipitation and quantitated in a γ-ray counter. Bound MPR 300 was calculated as percentage of tricliloroacctic acid-precipitable radioactivity in the eluate with 5 mM mannose 6 phosphate relative to total tricliloroacctic acid-precipitable radioactivity recovered in all column fractions.

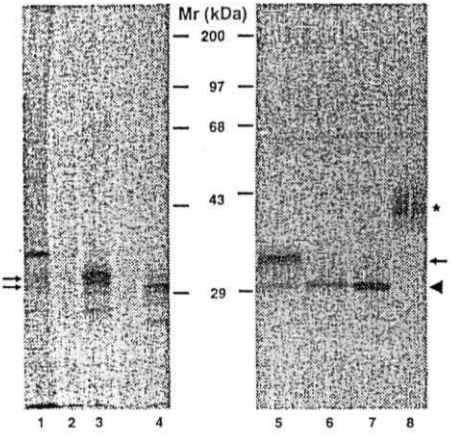

Fig. 4. Identification of MPR 46 in *Xiphophorus* cells. Left: A membrane extract of metabolically labelled *Xiphophorus* cells was subjected to PM-Sepharose affinity chromatography and MPR 46 from the column fractions was immunoprecipitated with anti-MSC1 antibody. Conditions are described under methods; the immunoprecipitates were analysed by 10% SDS-PAGE under reducing (lanes 1-3) or non-reducing (lane 4) conditions followed by fluorography. Lane (I) flow through; (2) glucose 6-phosphatc eluate; (3, 4) mannose 6-phosphate eluale. Right: 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 immunoprecipitation with anti-MSC1 antibody. The immunoprecipitate was split into two aliquots and treated for 16 h with (lanes 6 and 7). or without (lanes 5 and 8) PNGase F followed by SDS-PAGE (10% gel) and fluorography. Arrows: fish MPR 46, asterisk: chicken MPR 46, arrowhead: deglycosylated MPR 46.

We also searched for MPR 300 **in** the *Xiphophorus* cell line. Our failure to detect MPR 300 in metabolically labelled cell extracts may be due to lack of cross-reactivity with antibody against **mammalian** MPR 300 or due to loss of expression in the immortalized cell line, as observed for certain mammalian cells [6].

In summary, this report is the first to show the existence of both MPRs in the earliest non-mammalian vertebrates, fish. The identification of the proteins as MPRs was established by their ability to bind to PM-Sepharose and cross-reactivity with antibodies that have been recently shown to immunoprecipitate MPR 46 proteins from both reptiles and amphibians [18].

Our studies confirm that both MPRs are consistently present among all vertebrate species (non-mammalian and **mammalian)** and similarly bind to PM-Sepharose as do the receptors from mammals. **It remains** to be established whether these proteins have functionalities and sequences similar to those of their **mammalian** counter parts, a future direction of work.

5. Unlinked list

AUTHOR PLEASE CITE Ref. [14] IN THE TEXT.

Acknowledgements

SKN and YUL thank the Deutscher Akademischer Austauschdienst, Bonn, Germany for the support given to YUL (grant: A/97/00801). Research work in the laboratory of NSK is supported by Department of Science and Technology, New Delhi, India (SP/SO/D21/97). Research work in the laboratory of KvF is supported by Deutsche Forschungsgemeinschaft (SFB 236/525).

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