Molecular cloning, sequencing of fish Mannose 6-phosphate receptors (MPR 300 and 46) and identification of MPR 300 like proteins in Arthropoda and Annelidae

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that this thesis entitled **"Molecular cloning, sequencing of fish Mannose 6-phosphate receptors (MPR 300 and 46) and identification of MPR 300 like proteins in Arthropoda and Annelidae** submitted to the University of Hyderabad by **Mr. VEGIRAJU SURYANARAYANA RAJU** for the degree of Doctor of Philosophy, is based on the studies carried out by him 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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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.** (Part of the work has also been carried out in Muenster, Germany). 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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ABBREVIATIONS

APS	Ammoniumperoxodisulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bp	base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
cpm	Counts per minute
CTP	Cytidine triphosphate
dATP	Deoxyadinosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DVS	Divinyl sulfone
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
FCS	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-I1	Insulin like growth factor-Il
kb	Kilo basepair
kDa	Kilo Dalton
LB	Luria Bertani
Man 6-P	Mannose 6-phosphate
min	Minute
MOPS	Morpholino propane sulfonic acid

MPR	Mannose 6-phosphate receptor
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/ Streptomycin
pH	-log (H^+) concentration
PM	Phosphomannan
pmol	Picomole
PMP	Pentamannosyl phosphate
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
SDS	Sodium dodecyl sulfate
Taq	*Thermophilus aquaticus*
TBS	Tris-buftered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylendiamine
TFA	Trifiuroacetic acid
TGN	trans-Golgi network
Tris	Melting temperature
Tris	Tris-(Hydroxymethyl) aminoethane
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UV	Ultraviolet
β-M.E	β– mercaptoethanol
µCi	micro Curie

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CHAPTER I

INTRODUCTION

Cells produce many proteins which act at specific destinations or tissues. These proteins are to be transported from the site of synthesis to acting site through specific transport systems. Studies on these transport systems have been carried out in various organisms like bacteria, yeast, plant and animal cells. The receptor mediated trafficking of acid hydrolases to lysosomes was the best understood pathway in which these enzymes are transported from endoplasmic reticulum to their destinations (von Figura & Hasilik,1986; Pfeffer,1988; Kornfeld & Mellman,1989; Kornfeld,1992; Hille Rehfeld,1995; Pohlmann, 1996).

Intracellular transport of lysosomal enzymes :

Lysosomes are intracellular organelles that contain many hydrolytic enzymes which aid in the degradation of internalized and endogenous macromolecules. The intracellular transport of 50 newly synthesized lysosomal enzymes to lysosomes are mediated by mannose-6-phosphate receptors (MPRs).

Lysosomal enzymes are synthesized on membrane bound ribosomes in the endoplasmic reticulum and are co-translationally glycosylated at selected asparagine residues. Lysosomal enzymes acquire mannose-6-phosphate (M6P) residues that act as recognition signals for MPR's to bind and target them to lysosomes. Generation of this M6P signal occurs in two steps (Fig.l), first the phosphotransferase binds a phosphorylated N-acetyl glucosamine (GlcNAc) moiety to the C-6 hydroxyl group of mannose to form the M6P-oGlcNAc phosphodiester intermediate. Second, in the *trans-golgi* network (TGN) the uncovering enzyme removes the GlcNAc moiety there by generating the M6P signal for recognition by the MPR's.

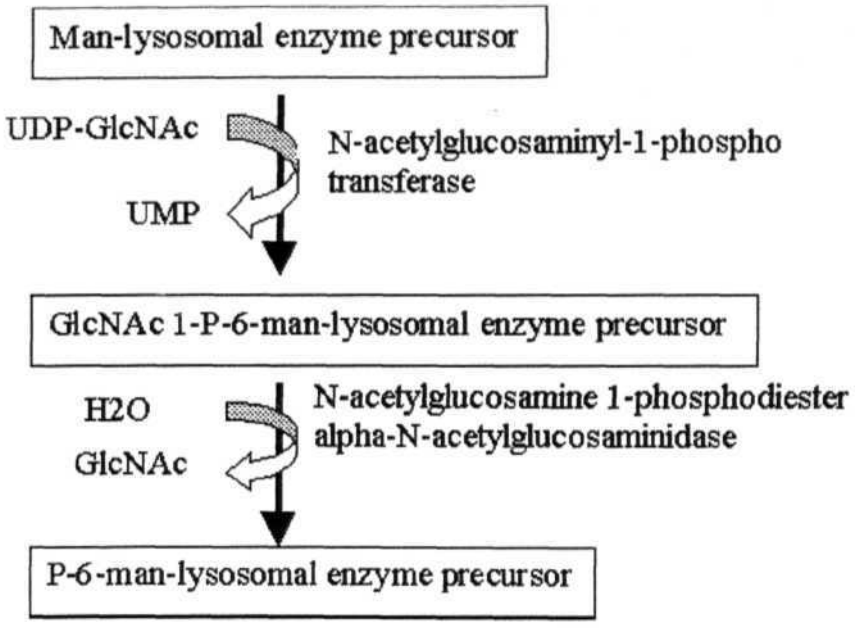


Figure 1: Two step biosynthesis process for the generation of terminal mannose 6-phosphate recognition marker in lysosomal enzymes

After the M6P recognition by the MPRs in TGN, the enzyme receptor complex is transported to a prelysosomal compartment, where the low pH environment induces the release of the enzyme from the receptors. The enzymes are packaged into the lysosomes and the receptors are recycled back to TGN to repeat the process or move to the cell surface to internalize a diverse population of endogenous ligands.

Significance of M6P recognition marker :

Hickman & Neufeld (1972) proposed the role of M6P signal acting in the segregation of lysosomal enzymes following their studies on fibroblasts from patients of I-cell disease. I-cell disease/Inclusion cell disease/Mucolipodosis is an autosomal recessive lysosomal disorder. Cells of these patients are deficient in GlcNAc phosphotransferase activity necessary for generating M6P signal (Reitman,1981; Mueller, 1983). So the lysosomal enzymes that lack the M6P signal are secreted outside the cell instead of being sent to the lysosomes. As a consequence, various undigested materials accumulate in the lysosomes and these are called as the inclusion bodies. The disease effects the kidneys,

heart and nervous system due to progressive storage of undigested material and the patient dies of heart failure before reaching puberty (Saton,1983; Ludwig,1993; Kornfeld,1995).

Significantly, mouse fibroblasts lacking both MPRs show condition as in I-cell disease patients (Pohlmann,1995; Ludwig,1996; Dittmer, 1998) demonstrating that the same phenotype can be seen from the deficiency of M6P signal generation or lack of MPR's. The devastating effects of aberrant transport of lysosomal enzymes in I-cell patients underscores the importance of the M6P dependent lysosomal enzyme targeting pathway. The deficiency of lysosomal enzymes shows a group of about 40 different lysosomal storage diseases, each characterized by a specific lysosomal enzyme deficiency in a variety of tissues. The majority of these are inherited as autosomal recessive conditions although Hunter disease and Fabry disease are X-linked. Some of these diseases are shown in Table below.

DISORDER	ENZYME
Tay-Sachs disease, Sandhoff disease	(3-Hexosaminidase
Gaucher disease	P- Glucosidase
Krabbe disease	Galactocerebrosidase
Metachromatic leucodystrophy	Arylsulphatase A
Fabry disease	α-Galactosidase A
Farber disease	Ceramidase
Niemann-Pick disease	Sphingomyelinase
Hurler's disease	α-L-Iduronidase
α/β-Mannosidosis	α/β-Mannosidase
Fucosidosis	α-Fucosidase

Table 1: Some lysosomal storage disorders and their deficient enzymes.

MPRs are type I transmembrane glycoproteins which mediate transport of lysosomal enzymes in the cell. MPRs are designated as P-type lectins, based on the unique ability of these lectins to recognize phosphorylated mannose residues. Two MPRs constitute this P-type lectin family (DrickamerJ 993). The Cation dependent MPR/CD-MPR/MPR 46 which requires divalent cations for optimal binding to ligands, and Cation independent MPR/IGF II-MPR/MPR 300 that does not require divalent cations for optimal binding.

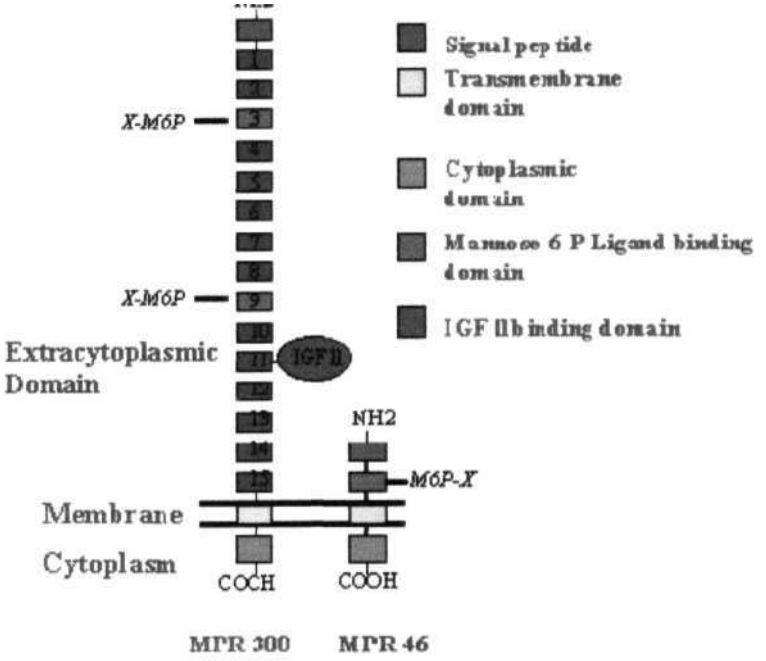


Figure 2: Schematic representation of the primary structure of MPR's

MPR 300 alone has been shown to be a multifunctional protein that binds to several M6P containing ligands and non M6P containing ligands that are listed in the **Table 2.**

Biochemical, morphological, immuno cytochemical and subcellular fractionation techniques have been employed to study the intracellular localization of MPR's. These studies have shown that the receptors are present in the plasma membrane (Sahagian, 1981), endosomes (Dickson, 1983), golgi membrane (Brown, 1984) and coated

vesicles (Sahagian,1985), but are not found in mature lysosomes.

M6P containing ligands	Consequence of binding to MPR 300
Lysosomal enzymes	Endocytosis and/or targeting to lysosomes
Transforming growth factor-P precursor (TGF-P)	Proteolytic activation at the cell surface
Leukemia inhibitory factor	Endocytosis and degradation in lysosomes
Proliferin	Induction of endothelial cell migration and angiogenesis
Thyroglobulin	Endocytosis and activation and/or degradation in lysosomes
Renin precursor	Internalization and proteolytic activation and/or degradation in lysosomes
Granzyme A	Targeting to lytic granules and role in apoptosis
Granzyme B	Internalization and rapid induction of apoptosis
Dnase 1	Targeting to lysosomes
CD 26	Internalization and role in T cell activation
Epidermal growth factor receptor	Endocytosis and degradation in lysosomes
Herpes simplex viral glycoprotein D	Facilitation of viral entry into cells and transmission between cells
Varicella-zoster viral glycoprotein I	Facilitation of viral entry into cells
Non M6P containing ligands	**Consequence of binding to MPR 300**
Insulin like growth factor II (IGF-II)	Endocytosis and degradation in lysosomes
Retinoic acid	Growth inhibition and/or induction of apoptosis
Urokinase-type plasminogen activator receptor (uPAR)	Role in activation of TGF-P precursor at cell surface; endocytosis and degradation in lysosomes
Plasminogen	Conversion to plasmin, which can activate TGF-P Precursor

Table 2 : Reported ligands for MPR 300.

MPR 46 :

This protein was first purified by affinity chromatography on phosphomannan gels from bovine and human liver tissue and was biochemically characterized (Stein *et al,* 1986). Further chemical modification data on the human receptor suggested the possible involvement of histidine and arginine groups in ligand binding.

Primary structure:

Hoflack & Kornfeld (1985) first discovered a cation dependent MPR with an apparent molecular mass of 46 kDa. Subsequently, cDNA clones for this receptor have been isolated and sequenced from human (Pohlmann *et al,* 1987), bovine (Dahms *et* a/, 1987), mouse (Ma,1991; Koster,1991) and rat (Kanamori *et* al,1998). Among the non-mammalian vertebrates, partial cDNA sequence of the chicken MPR 46 is also known (Matzner et al,1996).

Mammalian MPR 46 consists of 4 structural domains with 277 amino acids in humans. It consists of a 20 amino acid signal sequence at amino terminal region, an extracytoplasmic domain made of 170 amino acids , followed by a 20 amino acid transmembrane domain and a 67 amino acid cytoplasmic domain. The signal sequence directs translocation of nascent MPR 46 polypeptides across the ER membrane and is subsequently cleaved. The extracytoplasmic domain contains M6P binding site. The transmembrane domain anchors to the membrane and cytoplasmic domain extends into the cytosol which functions in receptor trafficking and contains a casein kinase II phosphorylation site.

Human MPR 46 is located on chromosome 12 (Pohlmann *et al,* 1987) and mouse MPR 46 on chromosome 6 (Ludwig *et* al, 1992). It spans around 12 kb and contains 7 exons. During development and in adult tissues different levels of mRNA were observed in

chicken (Matzner *et al*, 1996). Thus several lines of evidence indicate that MPR 46 expression is tissue specific and developmentally regulated.

MPR 46 has 5 potential N-linked glycosylation sites out of which 4 are used and located on extracytoplasmic domain (Wendland *et al*, 1991). These N-linked oligosaccharides promote proper folding of the receptor, which is essential for high affinity ligand binding and intracellular transport. Only single N-linked oligosaccharide is sufficient to facilitate proper folding.

The 6 cysteines that generate 3 intracellular disulfide bonds, essential for proper folding, (Roberts, 1998) are conserved among different species. Replacement of cysteine residues with glycine disrupts the folding in MPR 46 and the binding ability of M6P is also lost. MPR 46 exists as homodimer in membranes.

Post **translational modifications** :

Post translational modifications like phosphorylation and acylation, which are supposed to have a role in modulating the trafficking of the receptor, occur at carboxyl terminal cytoplasmic tail of MPR 46. *In vivo* studies showed that serine 57 in the cytoplasmic domain of bovine MPR 46 could be phosphorylated by casein kinase II and dephosphorylated by protein phosphatase 2A, (Korner,1994) suggesting that phosphorylation plays a key role in the intracellular transport of MPR 46. *In vitro* assays show that casein kinase II phosphorylation site mediates high affinity interaction and clathrin associated adaptor protein (AP) complex formation, (AP-1 with TGN membranes), suggesting a role for serine phosphorylation in the sorting of MPR 46 from TGN to endosomes (Mauxion *et al,* 1996).

Sorting of MPR 46:

Sorting of both MPR's from TGN to endosomes is mediated by clathrin associated golgi localized y-ear containing ADP-ribosylation factor binding (GGA) protein family

members rather than by AP-1 (Puertollano *et al*,2001; Zhu *et al*,2001; Takatsu *et al*,2001).

Acylation of MPR 46 involves the reverse palmitoylation of the two cysteine residues corresponding to positions 30 and 34 of bovine MPR 46 in its tail (Schweizer,1996). Cysteine 34 substitution with alanine resulted in loss of sorting of the lysosomal enzyme cathepsin D and accumulation of MPR 46 in dense lysosomes. From the results it was assumed that palmitoylation might anchor the tail to the membrane and there by form a signaling loop necessary for enzyme sorting and receptor trafficking. Two aromatic residues phenyl alanine 18 and tryptophan 19 and the amino acids flanking around these are important and modifications of these resulted in change in the conformation of sorting loop which is important for sorting of MPR's in endosomes (Schweizer *et al*, 1997).

Crystallographic data of MPR 46:

X-ray crystallographic three dimensional structure of extracytoplasmic domain of MPR 46 contains one a helix and two β sheets. Crystal structure data confirmed the importance of H 105 and R 111 for carbohydrate recognition by MPR 46. Previous chemical modifications (Stein *et al*, 1987) and site directed mutagenesis studies using phosphomannan affinity chromatography (Wendland *et al*, 1991) revealed the importance of these residues in carbohydrate recognition. Recent site directed mutagenesis using pentamannosyl phosphate agarose affinity chromatography also demonstrates that the side chain interactions of 4 residues Q 66, R 111, E 133 and Y 143 which are all in proximity to form contacts with 2,3 & 4 hydroxyl groups of terminal mannose ring are critical for M6P recognition by MPR 46 (Olson, 1999). Substitutions of Y 45 and R 135 residues impaired M6P binding.

Crystal structure also revealed that the presence of Mn^{+2} cation presence enhances the binding of the phosphate group by shielding it from the negatively charged binding site residue D 103 (Roberts, 1998; Olson, 1999). Human and porcine MPR 46 has no influence on ligand binding due to the presence of divalent cations. Loop D encompassing residues E 134 - C 141 play a role in carbohydrate recognition (Olson,2002).

It was observed that the truncated MPR 46 containing only the extracytoplasmic domain retained the ligand binding affinity, confirming that the transmembrane and cytoplasmic domains are not required for ligand binding (Dahms, 1989; Wendland,1989; Causing 988).

MPR 300:

Primary structure :

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 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). MPR 300 is a type 1 transmembrane glycoprotein. It is also a member of P-type lectins.

Complete cDNA sequences of MPR 300 have been known for bovine (Lobel *et al,* 1988), human (Oshima *et al,* 1988), mouse, rat, chicken (Zhou *et al,* 1995), pig, rabbit, bat, colugo, hedge hog, ring tailed lemur, tree shrew, opossum, red necked wallaby, echidna and duck bill platypus. Partial cDNA sequences of sheep (AA G48349), fish (Udaya lakshmi *et* al,2000) kangaroo (Yandell *et* al,2000) and goat (Suresh et al., 2004) are also known.

Human MPR 300 is composed of a 40 amino acid signal sequence, 2265 amino acid extracytoplasmic region composed of 15 homologous repeat sequences (each having of 134-167 amino acids), a 23 amino acid transmembrane domain followed by 164 amino acid carboxyl terminal cytoplasmic domain. Extracytoplasmic domain contains two carbohydrate binding sites and one IGF-II binding site.

Complete genomic structure of MPR 300 has been analyzed in mouse (Szebenyi *et al,* 1994) and human (Killian *et al, 1999).* In mouse it is on chromosome 17 and in human on chromosome 6. Human gene is 136 kb and contains 48 exons. MPR 300 is tissue specific and developmentally regulated. It contains 19 potential N-linked glycosylation sites in extracytoplasmic domain and that help in proper folding of the receptor.

Post translational modifications :

Phosphorylation of threonine and tyrosine residues in the cytoplasmic tail of MPR 300 have also been observed, but only serine phosphorylation of the receptor is well studied. The cytoplasmic domain of bovine MPR 300 has 3 potential serine phosphorylation sites at positions 19, 85 and 156. Phosphorylation of 85 and 156 serine resides in casein kinase like motifs which along with TGN and clathrin coated vesicles play a role in trafficking of the receptor (Meresse,1993). Consistent with a role in receptor trafficking, serine phosphorylation mediated by protein kinase C or by okadaic acid inhibition of

sensitive phosphatases such as protein phosphatase 2A, appears to influence the amount of MPR 300 present at the cell surface (Hu,1990; Braulke, 1992; Zhang *et al, 1991).*

Instead of phosphorylation, acidic residues adjacent to serine 156 with in the carboxyl terminal casein kinase like site in conjunction with the dileucine motif are the critical components of the lysosomal enzyme sorting signal recognized by GGA protein family members in the TGN (Puertollano *et* a/,2001; Zhu *et* a/,2001; Takatsu *et* a/,2001; Chen *et al,* 1997).

MPR 300 is subjected to palmitoylation (Westcott,1988) and the functional significance of this modification or sites of attachment for MPR 300 is not known.

Recently it was found that ethanol consumption decreases the synthesis of the MPR 300 but does not decrease its mRNA (James Haorah *et al,* 2003).

Carbohydrate **recognition properties** of **MPR 300** :

Two carbohydrate binding sites are present in 3 and 9 domains of extracytoplasmic domain of MPR 300 (Westlund *et* al,1991; Dahms *et* a/,1993). R-435 in domain 3 and R-1334 in domain 9 are critical for ligand binding. Site directed mutagenesis studies combined with pentamannosyl phosphate agarose chromatography and binding affinity analysis confirmed the sequence predictions, identifying four residues Q 392, S 431, E 460 & Y 465 in domain 3 and four residues Q 1292, H 1329, E 1354 & Y 1360 in domain 9 as essential for carbohydrate recognition by bovine MPR 300 (Dahms and Hancock,2002). Carboxyl terminal M6P binding site in domain 9 of MPR 300 exhibits optimal binding at pH 6.4 - 6.5 and in domain 3 at 6.9 -7.0.

MPR 300 binds to lysosomal enzymes from *Dictyostelium Discoideum* which contain mannose-6-sulfate residues and small phospho diester M6P methyl ester(M6P-OCH_3) instead of M6P phospho monoester (Gabel,1984; Freeze, 1986). Carbohydrate binding site in domain 9 is specific for phosphor monoester, M6P and in domain 3 for M6P-

OCH_3 phospho diester and mannose-6-sulfate with 10 and 20 fold lower affinity than M6P (Marron-Terada, 2000).

IGF-II recognition by MPR 300 :

MPR 300 from viviparous mammals binds to non-glycosylated polypeptide hormone IGF II (Morgan, 1987; Mac Donald, 1988; Tong,1988) at a binding site on domain 11, corresponding to residues 1524-1590 in humans. Only a single residue I 1572 in domain 11 is important for IGF II binding as revealed by mutagenesis studies in which substitution of isoleucine with threonine eliminates IGF II binding (Garmroudi, 1996; Byrd,1999; Devi, 1999). The inability of MPR 300 from platypus, chicken and frog to bind IGF II have been attributed to significant alterations in their amino acid sequence in the amino terminal portion of domain 11 as compared to viviparous mammals (Zhou *et* al, 1998; Killian *et* al,2000). Although these findings suggest that IGF II binding is confined to viviparous mammals, where as carbohydrate recognition function of the receptor is widely utilized by mammalian as well as non-mammalian species. Mendez et al, (2001) have provided the first demonstration of binding of MPR 300 from a non-mammalian vertebrate fish to IGF-II. Therefore, the extent with which IGF II binding site is expressed in the MPR 300 among various species remains to be fully defined.

Sequence elements within domain 13 contribute a 5-10 fold enhancement to the binding affinity of the receptor for IGF II (Grimme,2000; Devi, 1998; Linnell,2001). In domain 13, a 43 residue insertion is present that exhibits 50% sequence identity to the fibronectin type II domain. This sequence in MPR 300 is responsible for IGF II binding affinity enhancement associated with domain 13 (Devi, 1998).

MPR 300 receptors from embryonic bovine tracheal cells and embryonic human skin fibroblasts have been shown to contain covalently bound palmitic acid, most likely in

an amide linkage (Westcott,1988). The functional significance of this modification is unknown.

Subcellular localization and trafficking of the MPR's :

Recognition of the M6P signal found on newly synthesized acid hydrolases by the P-type lectins occurs in TGN, there by selectively sorting the lysosomal enzymes from other proteins in the secretory pathway (Le Borgne,! 998; Rohrer,2001).

The enzyme bound receptor complexes are subsequently transported from the TGN to endosome compartments via clathrin coated vesicles. Although a role for AP-1 in the trafficking of MPRs from TGN to endosome has not been ruled out, several recent studies have evidence that members of clathrin associated GGA protein family rather than AP-1 bind to TGN sorting signal present in the cytoplasmic tail of the receptors (Puertollano *et* al,2001; Zhu *et* al,2001; Takatsu *et al,* 2001).

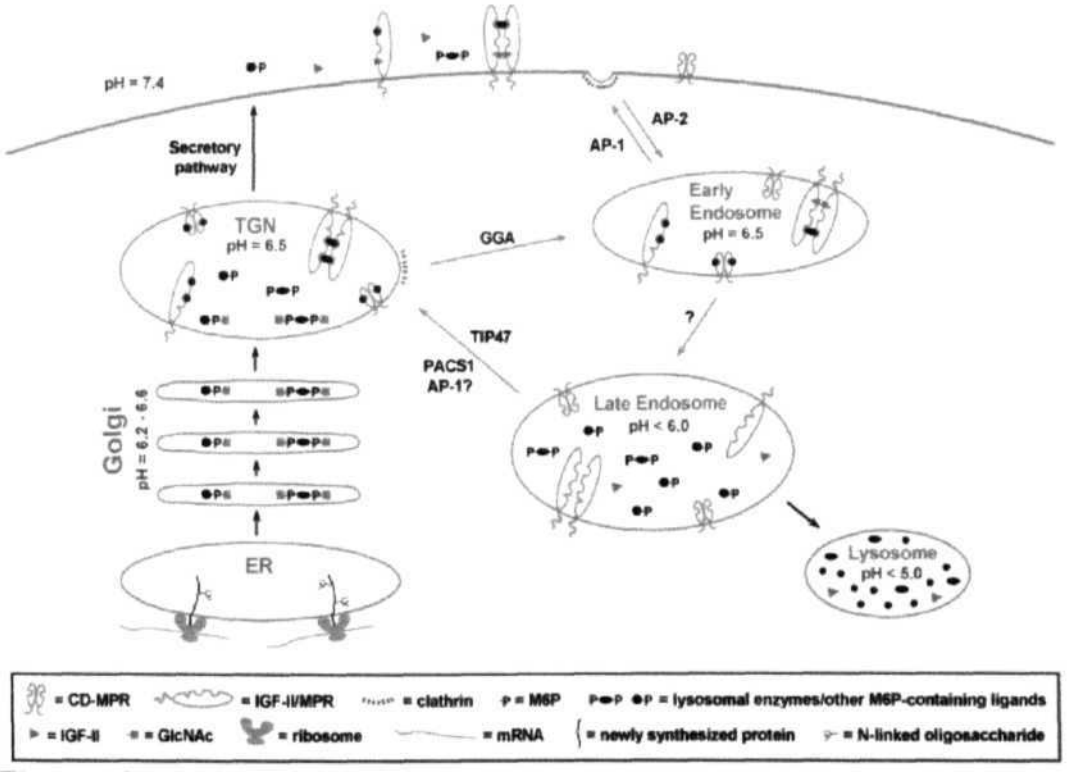


Figure 3 : A schematic representation of the subcellular localization and trafficking of the MPRs (Dahms N M and Hancock M K, 2002)

In both MPRs the TGN sorting signal is comprised of a cluster of predominantly acidic residues followed by a dileucine motif found near the carboxyl terminal of cytoplasmic domain ,that is recognized by the VHS domain of the GGA family members.

With in the low pH<6 the enzymes are released from MPR's and packed into lysosomes (Mullins,2001; Le Borgne, 1998). The MPRs do not travel to lysosomes but rather recycle from endosomal compartments back to the TGN.

It has been shown for the MPR 46 that a diaromatic endosomal sorting signal comprised of phenyl alanine 18 and tryptophan 19 in the cytoplasmic tail of bovine MPR 46 prevents its mislocalization to lysosomes (Schweizer,1997).

MPR 46 is recognized by tail interacting protein of 47 kDa (TIP 47) that in combination with Rab 9 GTPase is required for transport of the MPR's from endosome to the TGN, which is studied *in vitro* and *in vivo* (Lombardi,1993; Diaz, 1998; Corroll,2001; Barbero,2002). The region of the MPR 300 that is recognized by TIP 47 is highly conformational dependent and localized to residues 48-75 of the tail in human MPR 300 (Orsel,2000). AP-1 may play an important role in endosome to TGN receptor trafficking rather than sorting at the TGN.

Phosphoflirin acidic cluster sorting protein 1 (PACS1) a protein that binds *in vitro* to carboxyl terminal cluster of acidic residues in the cytoplasmic tail of MPR 300 in addition to interacting with AP-1 has impaired recycling in its absence. Thus, PACS1 may act as a connector between the MPR's and AP-1 to facilitate recycling of the receptors to the TGN (Wan,1998; Crump,2001).

Properties of MPR proteins at the cell surface :

At the cell surface as well as at pH 7.4, only MPR 300 binds a diverse population of M6P containing ligands and non-glycosylated polypeptide hormone IGF II. The role of MPR 46 at surface remains unclear, although results show over expressed MPR 46 in

namster kidney epithelial cells and mouse L cells suggest that its trafficking to the cell surface may be a mechanism for regulated secretion of M6P containing ligands into the extracellular milieu (Chao,1990).

Recent experiments by Nakagawa *et* al.,(2000) demonstrated that a novel micro tubule dependent motor from the kinesin super family of proteins KIF13A, transports MPR containing vesicles to the plasma membrane via interactions of KIF13A with the AP-1 complex.

Endocytosis of the MPR's on the other hand appears to primarily involve formation of clathrin coated vesicles mediated by the AP-2 complex. A single tyrosine based internalization sequence YKYSKV residues 24-29 has been identified in the cytoplasmic domain of MPR 300 (Lobel,1989; Canfield,1991; Jadot,1992) and evidence for AP-2 binding at this sequence has recently been presented (Orsel,2000). For internalization of MPR 46 the sequence phe-pro-his-leu-ala-phe residues (at 13-18 position) and the sequence tyr-arg-gly-val residues (at 45-48 position) are important (Johnson, 1990).

Consistent with the findings of multiple trafficking signals present in the MPR 46 *in vitro* biosensor studies and yeast two-hybrid experiments indicate that recognition of the cytosolic tail of the MPR 46 by the hetero tetramer AP-1 and AP-2 complexes involves multiple sequence elements (Honing, 1997; Storch, 2001).

The role of cell surface MPR 300 in facilitating the proteolytic activation of transforming growth factor p (TGF- p) has been extensively studied in recent years (Dennis,1991; Munger,1997).

TGF- β a critical cytokine that regulates the cellular differentiation and proliferation of many cell types is secreted from cells and stored in the extracellular matrix as an inactive precursor complex that necessitates an activation step to release the active form

of the growth factor (Munger, 1997). Activation of TGF- β precursor has been achieved using a variety of experimental conditions like extremes of pH or heat, chaotropic agents, low dose gamma irradiation and proteolysis. Recent histopathology studies by Crawford et al., (1998) indicate that thrombospondin-1 is a major *in vivo* activator of TGF- p. Several lines of evidence support an *in vivo* role for a proteolytic activation pathway involving binding of mannose-6-phosphorylated TGF- β precursors to cell surface MPR 300 and subsequent plasmin mediated cleavage of the precursors to active growth factors.

Recent data which demonstrated the ability of plasminogen and urokinase type plasminogen activator receptor (uPAR) to bind to the MPR 300 at regions of the receptor distinct from the M6P binding pockets and IGF II binding site suggest a possible model in which binding of urokinase plasminogen activator to uPAR that is complexed to MPR 300 facilitates conversion of plasminogen to plasmin which in turn proteolytically activates receptor bound TGF- β precursor molecules (Godar,1999; Nykjaer,1998).

The ability of the MPR 300 to recognize many functionally distinct ligands illustrates ;he multi functional nature of this receptor and its involvement in a variety of important physiological pathways.

IGF II plays a crucial role in mammalian growth including heart development, by influencing fetal cell division and differentiation (O'Dell,1998). The potency of this mitogenic factor was demonstrated by gene targeting studies that produced viable IGF II deficient mice that were 40% smaller than their wild type siblings (De Chiara,1990). Over expression of IGF II is observed in various human cancers and over growth syndromes (Werner,1996; Ellis,1998; Eggenschwiler,1997) and also plays a key role in the development of atherosclerosis in a recently studied mouse model (Zaina,2002).

IGF II recognition by the MPR 300 is postulated to be a general mechanism used to modulate circulating levels of the hormone by targeting it for degradation in lysosomes (Oka, 1985). MPR 300 deficient mice exhibited elevated levels of circulating IGF II and their organs were 25-30% larger in size than in their wild type litter mates (Lau, 1994).

Role of **MPR 300** as a tumor suppressor :

The ability of MPR 300 to regulate targeting of lysosomal enzymes to the lysosome, facilitate activation of the growth inhibitor TGF-p and modulate circulating levels of the potent cytokine leukemia inhibitory factor due to its M6P recognition function in addition to binding and targeting IGF II for degradation suggests that the MPR 300 plays an important role in tumor suppression. Loss of MPR 300 function is associated with progression of tumorigenesis (Oates, 1998; Jirtle *et al*, 1999; DaCosta, 2000). Single point mutation P2379T in the cytosolic tail has been identified, 9 human cancer associated missense mutations of the extracytoplasmic region of the MPR 300 have been reported and characterised in which receptors have altered M6P or IGF II binding properties (Byrd, 1999; Devi, 1999) supporting the hypothesis that loss of normal MPR 300 function contributes to carcinogenesis. Cell type and tissue specific role of the MPR's during development and in various disease states, typified by the recent report of a potential pathogenic role for elevated MPR 46 expression in sporadic Alzheimers disease (Mathews, 2002) requires further screening.

M6P independent pathway:

Neufeld and Mekusick (1983) have identified in I-cell patients that some lysosomal enzymes like β-glucocerebrosidase and cathepsin D use M6P independent pathway for their transport. This was 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 M6P signal (Lippincott-Schwarz,1986; Waheed,1988) and are transported to lysosomes, independent of M6P recognition system.

Sorting of lysosomal enzymes lacking M6P in the unicellular trypanosomes is thought to occur independent of M6P (Oeltmann,1994; Heute-Ferez,1999). M6P independent trafficking of the lysosomal proteases cathepsin D and L in mammalian cells has also been reported (Mclntyre,1993).

Yeast *Saccharomyces cerevisiae* is known to contain large vacuoles, which are functionally similar to mammalian lysosomes in many ways. Two enzymes from *Saccharomyces cervisiae* have been reported to contain peripheral mannosyl phosphate and mannobiosyl phosphate in diester linkage to high mannose type oligosaccharides. The vacuole enzyme carboxy peptidase-Y (CY) and invertase enzyme exhibit M6P independent pathway for segregation into vacuoles (Trimble, 1977).

α-L-Fucosidase :

a-L-Fucosidase is an ubiquitous glycosidase that has been studied in a variety of organisms including bacteria. In human pathology the importance of fucosidase is mainly associated with the neurovisceral storage disease, fucosidosis (Warner,1983; Johnson,1991). It was also reported in invertebrate *unio* in which 2 forms of a-L-Fucosidase one soluble and other bound to the sperm membrane are present in the gonads which have an apparent molecular mass of 56 and 68 kDa respectively on SDS-PAGE (Focarelli *et al*,1997). Recently the soluble enzyme was purified in our laboratory and antibodies for the same raised. Preliminary studies indicated that it

shows interaction with the MPR 300 protein purified from the same species suggesting that this enzyme is possibly lysosomal in origin. Since we detected MPR 300 like proteins from arthropoda and annelidae in the present study, we wanted to analyze if a mollusc related enzyme is present in the arthropods.

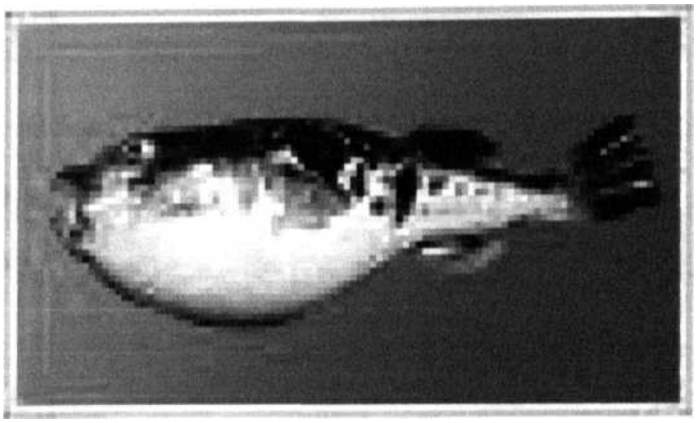
OBJECTIVES OF THE PRESENT WORK

The laboratory where this work was undertaken has been interested on the evolution of Mannose 6-phosphate receptor proteins. Alternate affinity methods were developed for the large scale isolation and purification of both MPR proteins from goat liver. Antibodies for these proteins were raised in rabbits and an ELISA method was developed to quantify the receptors. Recently the laboratory also obtained full length cDNA clone for the goat MPR 46 and a partial cDNA clone for the MPR 300. Employing the affinity matrices developed and using the antibodies several significant contributions have been made towards understanding the appearance of the putative receptors in the animal kingdom. In an earlier study, the fish receptors were identified and a partial cDNA clone for the fish MPR 300 protein was obtained. Recently the putative receptors were also identified from the invertebrate mollusc *(unio).* The following objectives have been put forward to undertake the present study.

1. Obtain full length cDNA sequence for fish MPR 300.
2. Obtain full length cDNA sequence for fish MPR 46.
3. Identification of MPR proteins in other invertebrates, such as the arthropoda and annelidae.
4. Identification and purification of α-fucosidase enzyme from the arthropods.

CHAPTER II

MOLECULAR CLONING AND SEQUENCING OF FISH MPR 300



FUGU RUBRIPES (Puffer fish)

PHYLUM	**CHORDATA**
SUB PHYLUM	**VERTEBRATA**
CLASS	**ACTINOPTERYGII**
DIVISION	**TELEOSTEI**
SUPER ORDER	**ACANTHOPTERYGll**
ORDER	**TETRAODONTIFORMES**
FAMILY	**TETRAODONTIDAE**

INTRODUCTION:

It is well established that the mammalian MPR 300 is a multifunctional receptor with high affinity binding site for mannose-6-phosphate in domain 3 and 9 (Dahms *et al,* 1993; Westlund *et al,* 1991) and an additional high affinity binding site for IGF-II in domain 11 (Schmidt *et al,* 1995). In contrast, chicken and frog MPR 300 fail to bind IGF-II (Clairmont *et al,* 1989; Canfield *et al,* 1989). Binding and endocytosis of IGF-II by MPR 300 contributes to the regulation of embryonic development of mice. It was also reported that mammalian MPR 300 also binds retinoic acid, human DNAse 1 (Kang *et al,* 1997; Cacia *et al,* 1998), but the physiological significance of these properties is not known yet.

The cDNA sequence studies of mammalian and chicken MPR 300 suggests that the receptors share structural properties, particularly the M6P binding regions being highly conserved (Zhou *et al,* 1995). Both putative receptors (MPR 300 and MPR 46) were first purified and characterized from our studies (Siva kumar *et al,* 1999).

In a recent study we have also isolated a partial cDNA clone for the fish MPR 300 that shows sequence similarity to the other known vertebrate MPR 300 proteins (Udaya lakshmi *et al,* 2000). In addition work in our laboratory on the molecular cloning of goat receptors (MPR 300 and 46), another representative species of mammals, indicates that their sequence structures are similar to other mammalian receptors (Suresh *et al,* 2004)

To further understand the evolution of the receptor proteins, it is necessary to obtain the sequence information of both the receptors from the earliest vertebrate fish. Therefore in the present study to obtain the full length cDNA clones for fish MPR 300, two approaches were used. The first approach was to do a cDNA library screening of the fish cDNA library using mouse MPR 300 cDNA probes and second was RT-PCR approach.

MATERIALS

Instruments.

Eppendorf thermocycler	
Transilluminator Model IL-400-M	Bachofer, Reutlingen
UV-Hand lamp (312 nm and 254 nm)	Bachofer,Reutlingen
373 A DNA sequencing system	Applied Biosystems

Chemicals:

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

Kits Used for Molecular Biological Work:

• QIAgen plasmid mini kit	Qiagen
• QIAgen plasmid midi kit	Qiagen
• QIAquick gel extraction kit	Qiagen
• TA cloning kit	Invitrogen
• Hot star *Taq* polymerase PCR QIAgen kit	Qiagen
• Dye terminator cycle sequencing kit	Applied Biosystems
• Random primer DNA labeling kit	Amersham,
▪ QIAgen Phage DNA isolation kit	Qiagen
• QIAgen RNeasy total RNA isolation kit	Qiagen
▪ First strand cDNA synthesis kit	Qiagen

Enzymes for Molecular Biological Work:

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

Plasmid DNA Vectors:

pCR 2.1 Topo cloning vector	Invitrogen
PCR-XL Topo cloning vector	Invitrogen
DNA Standards:	
DNA-Ladder	GIBCO/BRL
X DNA/Hind-III standard	GIBCO/BRL

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.

50 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 70 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

88.2 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 55^0C. 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 µg/ 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 NaAc (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 uL 500 mM EDTA, pH 8.0
720 uL 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 2M 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. 2 M Mg^{2+} stock and 2 M glucose was 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 µm 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.

Methods

Screening of the Phage **Library:**

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

Agar plates (25 ml NZY-agar/10 cm or 70-80 ml NZY-agar /14 cm plates) without any antibiotic were prepared well in advance (they should be at least 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.

Preparation of Host **Cell** (LE392) Culture:

A single bacterial cell colony XL1 blue MRF 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, 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).

Dilution of the Phage Library:

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

Phage Infection:

50 µL of diluted phage and 50 µL of host cells (A_{600} =2 is 1 5x10^9cells /mL) were mixed and incubated at 37^0C for 15 min with rotation.

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 (37^0C) agar plates, incubated at 37^0C for a minimum of 6-9 hours. Number of plaques formed was counted after incubation.

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

Plating of phage infected bacteria:

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 maintained at 48°C) and was spread uniformly over 14 cm bottom agar plates (minimum 2 days old) preheated to 37^0C for minimum 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°C for a minimum of 2-3 hours to prevent the top agar from sticking to the hybond-N nylon membrane filters.

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 2 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 5 min. Filters were processed as follows: The lifted membranes were placed in a petridish containing denaturation buffer for 2 minutes with DNA facing upwards. Membranes were then shifted to neutralization buffer and kept in it for 5 minutes and membrane filters were then rinsed briefly (not more than 30 seconds) in 2 x SSC and 0.2M Tris-HCl pH 7.5, then blotted on 3 MM Whatman paper. Filters were air-dried, DNA was UV cross-linked for 30 seconds (auto crosslinker) at 120,000 μJ of UV energy. 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.

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 37^0C with rotation for about 2-3 hours. Two cylinders were used taking 10 membranes in each.

Hybridization:

^{32}P labeled DNA probe (1-2 $x10^6$cpm/mL of hybridization mix), salmon sperm DNA (100 μg / mL of hybridization mix) were denatured by heating for 5 minutes at 95°C and was added to prehybridization mix (20-30 mL/ 10 filters) followed by overnight incubation at 37^0C 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 removed and 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

Low stringency washing:

Once with 6 x SSC at room temperature for 10 min

Once with 6 x SSC at 55°C for 30 min

Once with 1 x SSC at 55°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).

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

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.

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 uL of SM buffer.

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 µL of the eluted phage (1000-2000 pfu) from 3rd screening diluted to 100 uX with SM buffer and 100 uL XL_1 blue MRF' 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°C 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°C. Titer was determined using the protocol mentioned earlier.

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.

Total RNA Isolation Using QIAgen Kit:

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°C. Autoclave for 15 min to remove any traces of DEPC.

- Lysis of tissue: Tissuel was 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 µL of (3-M.E that was added to the buffer before use.
- Homogenization: A homogenous suspension of the tissue 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 RW1 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 uL of buffer RPE.

Column was rewashed with 500 µL of buffer RPE and centrifuged for 2 min at 13,000 rpm to dry the RNeasy membrane (*lt is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent elution).Column transferred into a new sterile 1.5 mL collection tube (supplied). 50 µL of RNase free water was pipetted directly onto the membrane, allowed to stand for a minute and centrifuged for 1 min at 10,000 rpm to elute RNA

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.

Denaturing Agarose Gel Electrophoresis for RNA:

1 % Agarose gel:

Agarose (electrophoresis grade)	2 g
10x MOPS-buffer	20 mL
DEPC-water	180 mL

Boiled in microwave, cooled to 65°C, 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.

Agarose **Gel Electrophoresis** for **DNA:**

DNA fragments were subjected to agarose gel electrophoresis for resolution

Sample buffer (Loading buffer IV)	0.25 %(w/v) Bromophenolblue
	40 % (w/v) Saccharose in TAE
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.

Gel Documentation:

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

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 were 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 the known sequences.

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.

First-Strand cDNA Synthesis:

- 5 µg (in 8 µL) of total RNA isolated was taken in a sterile eppendorf tube, heat denatured at 65°C for 10 min, then chilled on ice.
- 5 uL 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 uL of DTT (200 mM) solution, 1 uX (40 pmoles) of specific anti-sense primer and the heat denatured RNA were added
- Above reaction mix was incubated at 37^0C for 1 hour.

• The completed first strand reaction product was heated at 90^0 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.

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

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 an 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.

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

Following **were used for** one **PCR** reaction:

10x **PCR** buffer	2 µL
dNTP Mix	0.4 µL
Primers 1.2 µL **each** of sense and anti-sense **primers**	
Hot star Taq **DNA polymerase**	0.2 µL
Template **DNA**	
(First strand cDNA (5 µL) or DNA)	1 µL

Master mix was prepared as above. Template DNA was added at the end, volume was made up to 20 uL with sterile water. The PCR reaction was carried out using the thermal cycler program shown in the Table 3. 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	α	4

Table 3 : Thermal Cycler Program

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 uL). 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.

1 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). 0.75 ml of buffer PE was added to the column and centrifuged for 1 min at 13,000 rpm. Flow through was discarded and the column centrifuged as above. Column was placed into a clean 1.5 mL microfuge tube. To elute DNA, 50 μl 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.

TA **Cloning (*Invitrogen*):**

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. Advantages using the kit are, one can eliminate any enzymatic modifications of the PCR product.

Topo cloning:

Gel purified PCR product	3.5 μL
Salt solution	0.5 μL
PCR 2.1 Topo vector	1.0 uL

The above mixture was incubated 5 minutes at room temperature for ligation.

Transformation:

Before Start:

- Water bath was equilibrated to 42°C.
- A vial of SOC medium was thawed and incubated at 37^0C.

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 μL of 40 mg/mL X-Gal (chromogenic substrate for β-galactosidase) 60 μL of 100 mM 1PTG (isopropylthiogalactoside-used to maximize the expression of genes cloned in expression vectors). Liquid was allowed to soak into the plates by incubating at 37^0C 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. To 3 μL of it 25 μL of One Shot competent cells (TOP10F' provided in the kit) were added and incubated on ice for 30 minutes. The remaining ligation mixture was stored at - 20°C. Heat shock was given exactly for 30 seconds in a 42°C 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 37^0C for 1 hour at 180 rpm in a rotary-shaking incubator. The vials with the transformed cells were placed on ice. 50 μL and 200 μL 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.

Cloning with PCR XL-TOPO vector was carried out following the instructions of the manufacturer.

Plasmid DNA Isolation (QIAprep plasm id DNA Isolation kit):

Bacterial colony (white) was picked up with a sterile toothpick and inoculated into 5 mL medium (LB medium containing 200 μg/ mL ampicillin). From an overnight culture plasmid DNA was prepared and digested with restriction enzymes. 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 uL buffer P2 was added and the tube was gently inverted 4-6 times (solution becomes viscous and slightly clear). 350 uL of buffer N3 was added and the tube was inverted immediately but gently 4-6 times to avoid localized precipitation (solution becomes cloudy), centrifliged 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, briefly centrifuged and flow through discarded. Column was 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.

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 μL to this, 1 uL of enzyme and 1 uL 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 37^0C for 1-2 hours. Product was analyzed by analytical agarose gel electrophoresis.

2' 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).

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

The following reaction was set up

DNA	Premix	Primer
250 ng	3 μL	10 pmoles

Volume was made upto 10μl with sterile water.

Thermo Cycler Program:

1. Denaturation	96°C	55 seconds
2. Annealing	50°C	45 seconds
3. Primer extension	60°C	4 minutes

Total number of cycles 25

Purification and Precipitation of **DNA :**

The sequencing PCR product was purified using QIAGEN dye terminator sequencing purifying kit. The screw of the column in the kit was turned 180° and the column was opened in the lower end. The column was centrifuged at 3000rpm for 2 minutes and the flow through discarded. The column was placed in a sterile eppendorf tube and sequencing PCR product was added to the column and the column was spun at 3000 rpm for 2 minutes. The elute was collected and dried in speed vac for 3 min. Pellets were finally dissolved in sample buffer "BIG dye" (supplied in the kit), and electrophoresed and sequenced using 373 A DNA sequencing system according to the manufacturers manual.

Random Primer Labeling (Redivue random primer labeling kit):

25 ng of gel purified DNA probe was dissolved in 45 µL of sterile water. DNA was 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. (α ^{32}P) dCTP (2µL-5 µL (20-50µCi)) offered and incubated at 37°C for 10 min. The reaction was stopped by adding 150µl 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 µL) packed in 1mL blue tip, centrifuged for 2 min at 3000 rpm. Second elution was done with 100 µL of TE buffer. 1/100 of eluted volume was used for cerenkov counting.

% **Incorporation**

'x' cpm x 100 = Total counts.

Calculate total µCi (1 µCi = 2.22 x10^6 cpm)

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

Radioactivity offered = 1 µCi / mL of hybridization buffer used.

Northern Blotting (RNA Transfer):

Southern., (1975).

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 Overnight transfer was done at room temperature. Filter was air dried, exposed to UV light for 15 seconds, oven baked at 60°C for 1 hour and used for hybridization.

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;

After 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°C with an intensifying screen. Alternatively the membrane was also exposed to phosphorimaging screen and scanned after an overnight exposure.

RESULTS

Screening of fish cDNA library

To get the full length cDNA sequence of fish MPR 300 the cDNA library of *Xiphophorus maculatus* x *Xiphophorus hellari* hybrid was screened with a 1.25 kb fragment of Mouse MPR 300 radio labeled with random primer labeling kit.

Few clones were detected positive and they were screened up to tertiary screening.

From tertiary screened clones, 2 clones (Fig. 4) were processed and their sequences determined. The 2 clones sequenced are similar and exhibit the sequence for Ubiquitin gene.

Amplification of fugu MPR 300 cDNA by RT-PCR approach

The results with cDNA library screening were not promising, and hence, a second approach was used to obtain the full length clones. Here, total RNA was used as the starting material and by RT-PCR approach, first a 1.45 kb fragment (Fig.5) was amplified in the region of domain 8th to 11th of the receptor based on the genomic data available. It was cloned in to PCR 2.1 Topo vector and sequenced using vector specific primers. The sequence data showed homology with MPR 300 of other species. Subsequent PCRs using specific primers resulted in generation of several cDNA fragments, which cover the full length cDNA of fugu MPR 300. These were all cloned and sequenced. To construct the entire gene, three different clones were also isolated and the DNA sequenced (the results of these are not shown).

In addition, two large clones of 4.6 kb and 5.1 kb in PCR XL TOPO vector were also obtained using specific primers. These clones were isolated and kindly provided by Dr.B.Venkatesh, Singapore. These were completely sequenced in Hyderabad. These

Figure. 4 : Colony hybridization and screening strategy of phage clones (fish cDNA library constructed in *X ZAP* II phage DNA) with Mouse MPR 300 specific cDNA probe. Among the several clones identified in the primar screening step, only some of them were used for secondary and tertiar screening. As an example two of the clones were screened up to tertiary screening.

Figure. 5 : RT-PCR for the amplification of fugu MPR 300 1.45 kb fragment. RT-PCR product of fugu MPR 300 1.45 kb fragment obtained using gene specific primers designed from the database. The product was analyzed on 1% agarose gel electrophoresis. Arrow indicates the fragment. Lane 1, 1kb standard DNA ladder; Lane 2, 1.45 kb RT-PCR product of fugu MPR 300.

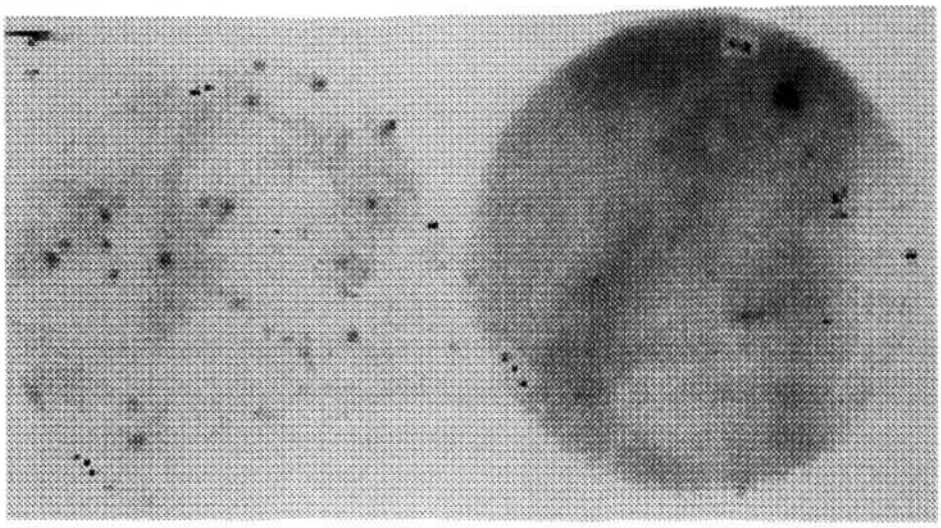


Figure. 4

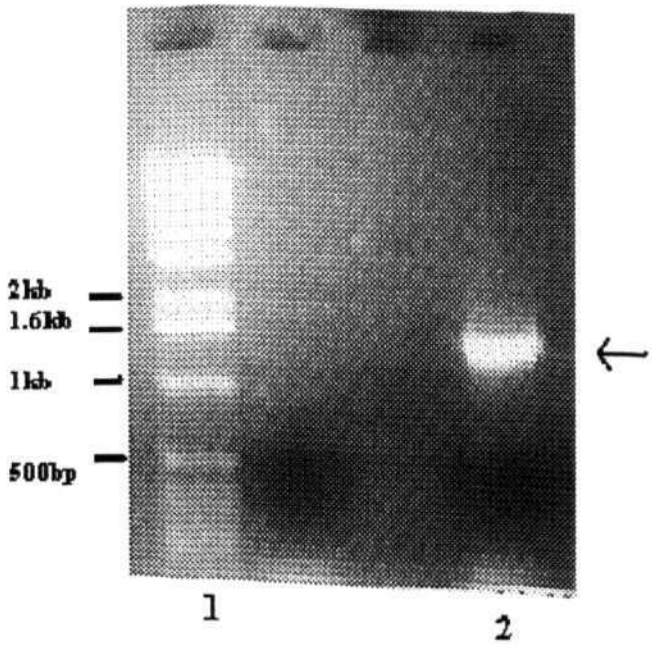


Figure. 5

clones (Fig.6) with overlapping sequences represent the entire length of the fugu MPR 300 gene. The list of primers used to obtain MPR 300 sequences are shown in Table 4

S.NO	Primer Sense(S)/ Antisense (AS)	Primer	Location Domain	Primer sequence
1	S	**RAJU300-1**	**5/6th**	ACGCTTGAACAGTACGACTTGTCC
2	S	RAJIJ300-2	12th	ATCAATGTCTGTCAGCCCCTCAAC
3	AS	RAJU300-3	15th	CTTGACCTGGCAGATGTTGGCG
4	AS	**RAJU300-4**	**CYT TAIL**	**AGGAGGTCCTCGTCACTGTCGT**
5	S	RAJIJ300-5	**1st**	CTCTGCAGCTACACATGGGAGGC
6	S	**RAJU300-6**	3rd	ATCAACTTTGAGTGCAACAAGACT
7	AS	**RAJU300-7**	3rd	AGTCTTGTTGCACTCAAAGTTGAT
8	AS	**RAJU300-8**	**5/6th**	GGACAAGTCGTACTGTTCAAGCGT
9	AS	**RAJU300-9**	10th	ATGACCTGTGGCGGGGTTGGAGA
10	S	RAJU300-10	13th	GCGGTGTGTCGAGTATCTGGTTCA
11	AS	**RAJU300-11**	**CYT TAIL**	CTGGATCACAAGCTCCCTTCTTTC
12	AS	**RAJU300-12**	**CYT TAIL**	TCACACCCTGAGGAGGTCCTCGTC
13	S	**RAJU300-13**	INI CODON	ATGTTATTTCGAGATAAACGCGG
14	S	RAJU300-14	57ATGXhol	CTCGAGGTAAACATGTTATTTCG
15	AS	**RAJU300-15**	**5/6th Xho1**	CTCGAGGGACAAGTCGTACTGTTC
16	AS	RAJU300-16	STOP **Xba1**	TCTAGATCACACCCTGAGGAGGTC
17	AS	3013R	7th	CTGGAGGCTCTTCTCAATCCCCAC
18	AS	3613R	8th	CCAGATGAACACGTACTCACAACC
19	S	3616F	8th	GAGTACGTGTTCATCTGGAGGACG
20	AS	5074R	12th	**GTTGAGGGGCTGACAGAC(T)ATTGAT**
21		UP SHORT		CTAATACGACTCACTATAGGGC

Table.4 : List of primers used to obtain fugu MPR 300 sequences.

CYT TAIL -cytoplasmic tail, INT CODON - initiation codon.

Primers used

1.45kb S-5'GAGTACGTGTTCATCTGGAGGACG 3'

AS-5'GTTGAGGGGCTGACAGACATTGAT 3'

2.4kb S-5'CTCGAGGTAAACATGTTATTTCG 3'

AS-5'CTCGAGGGACAAGTCGTACTGTTC 3'

3.25kb S-5'ATCAACTTTGAGTGCAACAAGACT 3'

AS-5'ATGACCTGTGGCGGGGTTGGAGA 3'

3.9kb S- 5'GAGTACGTGTTCATCTGGAGGACG 3'

AS-5'TCTAGATCACACCCTGAGGAGGTC 3'

4.6kb S-5'ATGTTATTTCGAGATAAACGCGG 3'

AS-5'ATGACCTGTGGCGGGGTTGGAGA 3'

5.1kb S-5'ACGCTTGAACAGTACGACTTGTCC 3'

AS-5'TCACACCCTGAGGAGGTCCTCGTC 3'

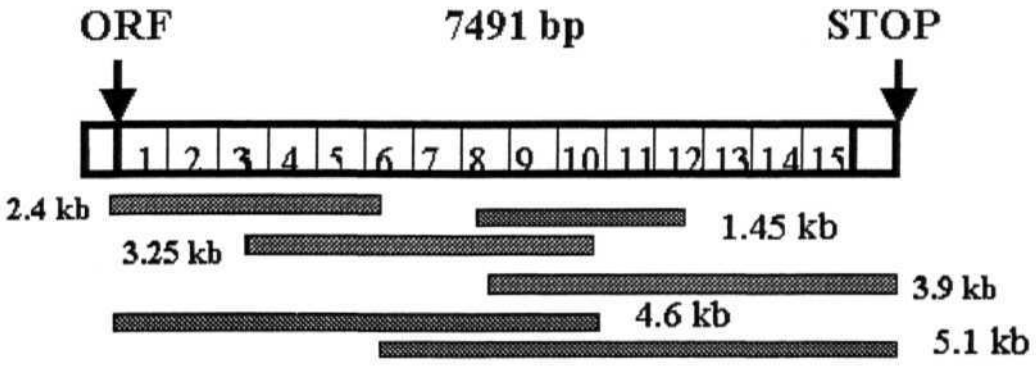


Figure. 6 : Strategy adopted to obtain different cDNA fragments of fugu MPR 300. The figure shows different cDNA fragments generated for fugu MPR 300 using the gene specific primer pairs as shown above. Arrow indicates the start codon and stop codon of the sequence. Clones 4.6 kb and 5.1 kb were prepared and given by Dr.B.Venkatesh, Singapore. (The DNA was completely sequenced in Hyderabad).

Sequence analysis of Fugu MPR 300

A 7564 bp cDNA sequence of MPR 300 gene was isolated from Fugu liver total RNA which consists of a 73 bp 5' UTR, 7488 bp ORF and a stop codon (Fig.7).

The cDNA sequence encodes for 2496 amino acids, which contains a 33 residue signal peptide, extra cytoplasmic domain in 15 repetitive domains **(Fig.8)**, transmembrane domain, and a cytoplasmic domain. It has 17 N-glycosylation sites and all the cysteine residues are highly conserved. It shows homology with MPR 300 from other species **(Fig.9)** and the ligand binding regions are conserved. The arginine residues in domain 3 and 9 critical for ligand binding are highly conserved among all the vertebrates.

However, the IGF-II binding site has a threonine residue in fish receptor as compared to the isoleucine in mammalian species. It remains to be established whether fugu MPR 300 can bind IGF-II.

Northern blot analysis

For Northern blot analysis total RNA was taken from different tissues and hybridised with 1.4 5kb fragment obtained by RT-PCR (this fragment showed extensive sequence homology to the mammalian MPR 300 proteins). The results show a band, which coincides with the MPR 300 RNA of other species **(Table.5)** detected earlier with a size of 9.5 kb **(Fig.10)**.

```
5'UTR AAGCAGTGGTAACAACGCAGAGTACGCG
      GGGGCTGTATCATGTCGTCCTAGTTTAGCTCCACGCACGGTAAAC
   1 atgttatttcgagataaacgcggccctcgtccgtctctgaagctg
       M  L  F  R  D  K  R  G  P  R  P  S  L  K  L
  46 cttctatggttattggccttcctggtatgttcgccagtccggtcc
       L  L  W  L  L  A  F  L  V  C  S  P  V  R  S
  91 gcggctgctggggacgacagcctgtggtaccgggacctctgcagc
       A  A  A▲ G  D  D  S  L  W  Y  R  D  L  C  S
 136 tacacatgggaggccatagataaagatagtaatgtcatctacacg
       Y  T  W  E  A  I  D  K  D  S  N  V  I  Y  T
 181 ctgaagctctgcgagtcctcgccaccaaccagctgtggtccagat
       L  K  L  C  E  S  S  P  P  T  S  C  G  P  D
 226 gtcgccgtgtgtgcccagaacctcggcaccaatacgaaccagtct
       V  A  V  C  A  Q  N  L  G  T  N  T N  Q  S
 271 gtcggtgatctgtctctgcagaagctctccggtcaggttctggac
       V  G  D  L  S  L  Q  K  L  S  G  Q  V  L  D
 316 ttcaacaccaccacaaaatgtcaggaaggcaacaacaccgttcag
       F  N  T  T T  K  C  Q  E  G  N  N  T  V  Q
 361 accagcttcagcttccagtgcgggaaaaccatggggacaccagag
       T  S  F  S  F  Q  C  G  K  T  M  G  T  P  E
 406 tttgtcgctgtgtctcagtgtgtgcattacttcgagtggaggacc
       F  V  A  V  S  Q  C  V  H  Y  F  E  W  R  T
 451 tacaccgcctgcaagaacaataagttcaagccacaaaaggaggtg
       Y  T  A  C  K  N  N  K  F  K  P  Q  K  E  V
 496 ccatgttacgtgttcgacacggacggcaagaagcacgacctcagc
       P  C  Y  V  F  D  T  D  G  K  K  H  D  L  S
 541 ccgttggtcaaagtgagcgacggcattctggtggacgacggtgac
       P  L  V  K  V  S  D  G  I  L  V  D  D  G  D
 586 gacagcatcgacttctacatcaacatctgccggagcctcaacctg
       D  S  I  D  F  Y  I  N  I  C  R  S  L  N  L
 631 cctggtaaatcctgtccagaaggttctgcagcctgcctggtcacc
       P  G  K  S  C  P  E  G  S  A  A  C  L  V  T
 676 agtcagggctccttcaacatagggtttcccaaaaagcggctggag
       S  Q  G  S  F  N  I  G  F  P  K  K  R  L  E
 721 ctgctctccaacgacagattgaggctgcagtatgaagtcgacgca
       L  L  S  N  D  R  L  R  L  Q  Y  E  V  D  A
 766 gattcctctcgtccagatatctgcaaagaacacgttccagctgtc
       D  S  S  R  P  D  I  C  K  E  H  V  P  A  V
 811 agcatcacattcgtctgcccatcacgcagatatcagggcagcagt
       S  I  T  F  V  C  P  S  R  R  Y  Q  G  S  S
 856 cctaaaatgacagcagattccagctgtcgatatgagatcgagtgg
       P  K  M  T  A  D  S  S  C  R  Y  E  I  E  W
 901 gtgaccgagtacgcctgtcaccgagactatttggagagccacagc
       V  T  E  Y  A  C  H  R  D  Y  L  E  S  H  S
 946 tgcacactgaacagcgagcagcacgatctgtccatcgacctaacg
       C  T  L  N  S  E  Q  H  D  L  S  I  D  L  T
 991 ccactcaccatggcctccacagatgttccatacagcgcgccctcc
       P  L  T  M  A  S  T  D  V  P  Y  S  A  P  S
1036 ggacccagcggcggagccgaaagctacatctactacctgaacgtg
       G  P  S  G  G  A  E  S  Y  I  Y  Y  L  N  V
1081 tgtggaaaagttgccaccgaagaatgtggcaaagagagtttcata
       C  G  K  V  A  T  E  E  C  G  K  E  S  F  I
1126 tcctcctgccaggtgaaggcgaccggagggctgtcgaaggtggct
       S  S  C  Q  V  K  A  T  G  G  L  S  K  V  A
1171 ggaagatacaggaaccagactctacggtattcagatggagatctg
       G  R  Y  R  N  Q  T  L  R  Y  S  D  G  D  L
1216 attttgatttacccaggtggatcccagtgctcctcaggctttgag
       I  L  I  Y  P  G  G  S  Q  C  S  S  G  F  E
1261 agaatgaccatcatcaactttgagtgcaacaagactgcatccaat
       R  M  T  I  I  N  F  E  C  N  K  T  A  S  N
1306 ggtgggcggggatctccagttttcactggcgagacggactgcact
```

```
     G  G  R  G  S  P  V  F  T  G  E  T  D  C  T
1351 tactactttaactgggagacggcgttagcctgtgcaaaagagaag
     Y  Y  F  N  W  E  T  A  L  A  C  A  K  E  K
1396 gaggatttgctgtgtcaagttcgggacgggaacaaacattacgac
     E  D  L  L  C  Q  V  R  D  G  N  K  H  Y  D
1441 ctttcaccacttacaagattccctggccccgaggccagtggaaac
     L  S  P  L  T  R  F  P  G  P  E  A  S  G  N
1486 tgggaggtagtggatgctcaatctccaaagtcggaatctcgtttc
     W  E  V  V  D  A  Q  S  P  K  S  E  S  R  F
1531 tatctgaacgtctgtcacaaggtggtccactcgggagctgctgtc
     Y  L  N  V  C  H  K  V  V  H  S  G  A  A  V
1576 ggctgccccgtaaatgcatcaatctgtgccgtggataagaaaaat
     G  C  P  V  N  A  S  I  C  A  V  D  K  K  N
1621 aatgccatcagtctgggcagctttctctcatctcctcagaagacc
     N  A  I  S  L  G  S  F  L  S  S  P  Q  K  T
1666 caaataggaagtgacatcagactcgtctattcagatggaagtttt
     Q  I  G  S  D  I  R  L  V  Y  S  D  G  S  F
1711 tgcaacagcaagaggaagcggatccgaaccatcctgacactgaag
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     C  K  P  G  D  L  E  S  A  P  I  L  R  S  I
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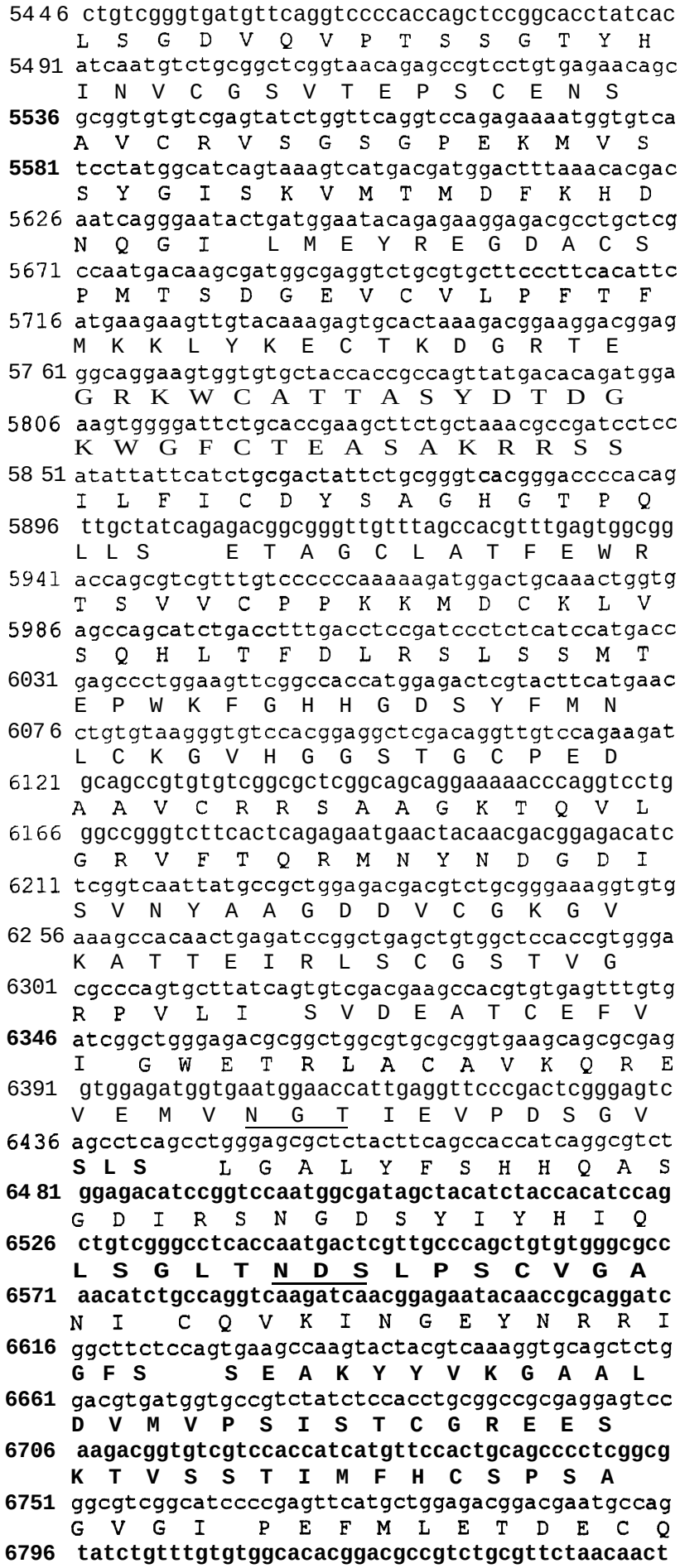
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      E  D  L  L  R  V  *
```

Figure.7 : Nucleotide and deduced amino acid sequence of full length fugu MPR 300 cDNA.

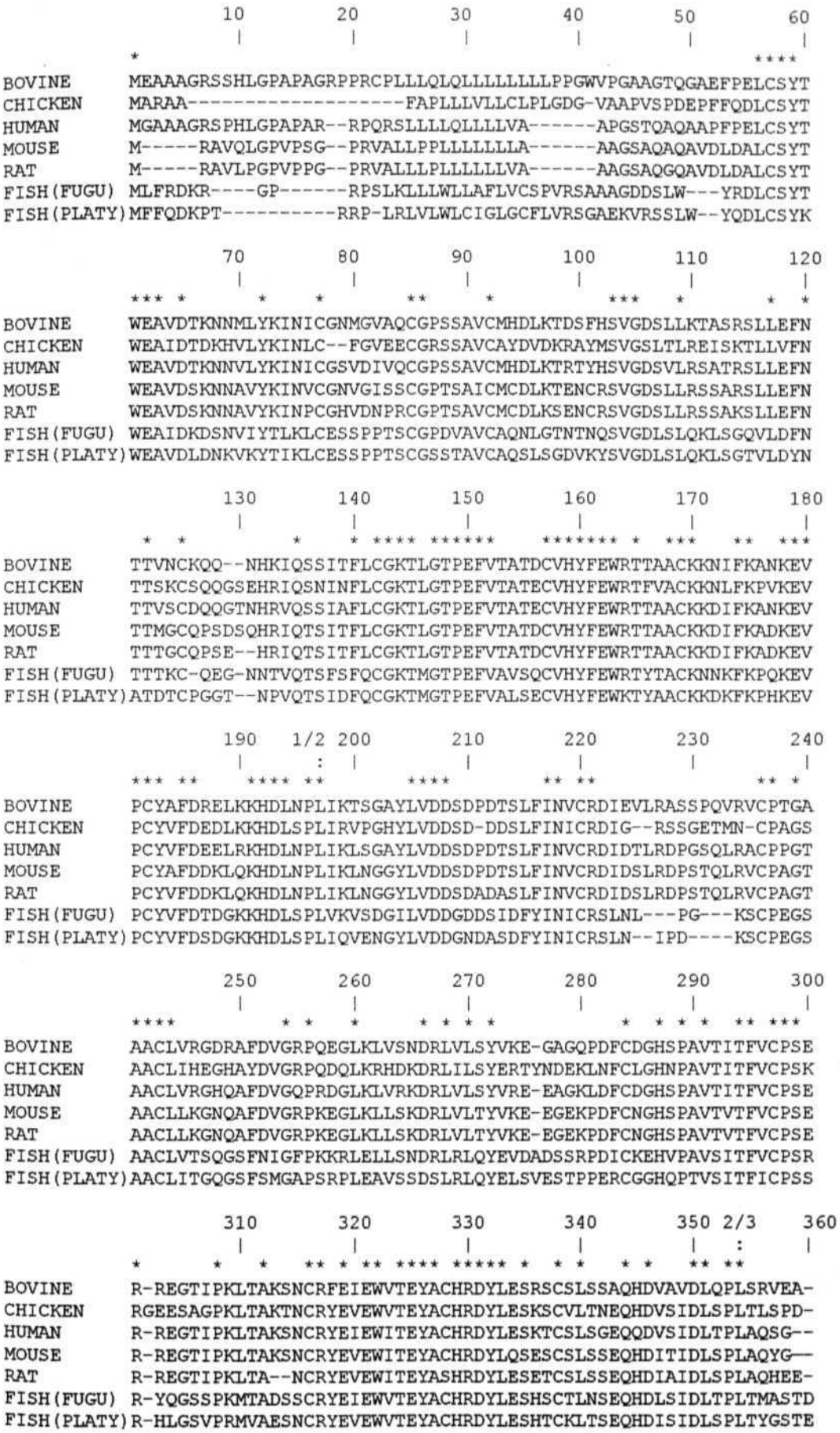
▲ indicates the signal peptide cleavage site. N glycosylation sites are under lined.

* indicates the stop codon.

Figure.8 : Alignment of amino acid sequence of 15 repetitive domains of fugu MPR 300.

! indicates the conserved cysteine residues.

*indicates the stretch of sequence in 13th domain that also aids in binding IGF-II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CHICKEN TTSKCSQQGSEHRIQSNINFLCGKTLGTPEFVTATECVHYFEWRTFVACKKNLFKPVKEV
HUMAN TTVSCDQQGTNHRVQSSIAFLCGKTLGTPEFVTATECVHYFEWRTTAACKKDIFKANKEV
MOUSE TTMGCQPSDSQHRIQTSITFLCGKTLGTPEFVTATDCVHYFEWRTTAACKKDIFKADKEV
RAT TTTGCQPSE--HRIQTSITFLCGKTLGTPEFVTATDCVHYFEWRTTAACKKDIFKADKEV
FISH(FUGU) TTTKC-QEG-NNTVQTSFSFQCGKTMGTPEFVAVSQCVHYFEWRTYTACKNNKFKPQKEV
FISH(PLATY) ATDTCPGGT--NPVQTSIDFQCGKTMGTPEFVALSECVHYFEWKTYAACKKDKFKPHKEV
190 1/2 200 210 220 230 240
BOVINE PCYAFDRELKKHDLNPLIKTSGAYLVDDSDPDTSLFINVCRDIEVLRASSPQVRVCPTGA
CHICKEN PCYVFDEDLKKHDLSPLIRVPGHYLVDDSD-DDSLFINICRDIG--RSSGETMN-CPAGS
HUMAN PCYVFDEELRKHDLNPLIKLSGAYLVDDSDPDTSLFINVCRDIDTLRDPGSQLRACPPGT
MOUSE PCYAFDDKLQKHDLNPLIKLNGGYLVDDSDPDTSLFINVCRDIDSLRDPSTQLRVCPAGT
RAT PCYVFDDKLQKHDLNPLIKLNGGYLVDDSDADASLFINVCRDIDSLRDPSTQLRVCPAGT
FISH(FUGU) PCYVFDTDGKKHDLSPLVKVSDGILVDDGDDSIDFYINICRSLNL---PG---KSCPEGS
FISH(PLATY) PCYVFDSDGKKHDLSPLIQVENGYLVDDGNDASDFYINICRSLN--IPD----KSCPEGS
250 260 270 280 290 300
BOVINE AACLVRGDRAFDVGRPQEGLKLVSNDRLVLSYVKE-GAGQPDFCDGHSPAVTITFVCPSE
CHICKEN AACLIHEGHAYDVGRPQDQLKRHDKDRLILSYERTYNDEKLNFCLGHNPAVTITFVCPSK
HUMAN AACLVRGHQAFDVGQPRDGLKLVRKDRLVLSYVRE-EAGKLDFCDGHSPAVTITFVCPSE
MOUSE AACLLKGNQAFDVGRPKEGLKLLSKDRLVLTYVKE-EGEKPDFCNGHSPAVTVTFVCPSE
RAT AACLLKGNQAFDVGRPKEGLKLLSKDRLVLTYVKE-EGEKPDFCNGHSPAVTVTFVCPSE
FISH(FUGU) AACLVTSQGSFNIGFPKKRLELLSNDRLRLQYEVDADSSRPDICKEHVPAVSITFVCPSR
FISH(PLATY) AACLITGQGSFSMGAPSRPLEAVSSDSLRLQYELSVESTPPERCGGHQPTVSITFICPSS
310 320 330 340 350 2/3 360
BOVINE R-REGTIPKLTAKSNCRFEIEWVTEYACHRDYLESRSCSLSSAQHDVAVDLQPLSRVEA-
CHICKEN RGEESAGPKLTAKTNCRYEVEWVTEYACHRDYLESKSCVLTNEQHDVSIDLSPLTLSPD-
HUMAN R-REGTIPKLTAKSNCRYEIEWITEYACHRDYLESKTCSLSGEQQDVSIDLTPLAQSG--
MOUSE R-REGTIPKLTAKSNCRYEVEWITEYACHRDYLQSESCSLSSEQHDITIDLSPLAQYG—
RAT R-REGTIPKLTA--NCRYEVEWITEYASHRDYLESETCSLSSEQHDIAIDLSPLAQHEE-
FISH(FUGU) R-YQGSSPKMTADSSCRYEIEWVTEYACHRDYLESHSCTLNSEQHDLSIDLTPLTMASTD
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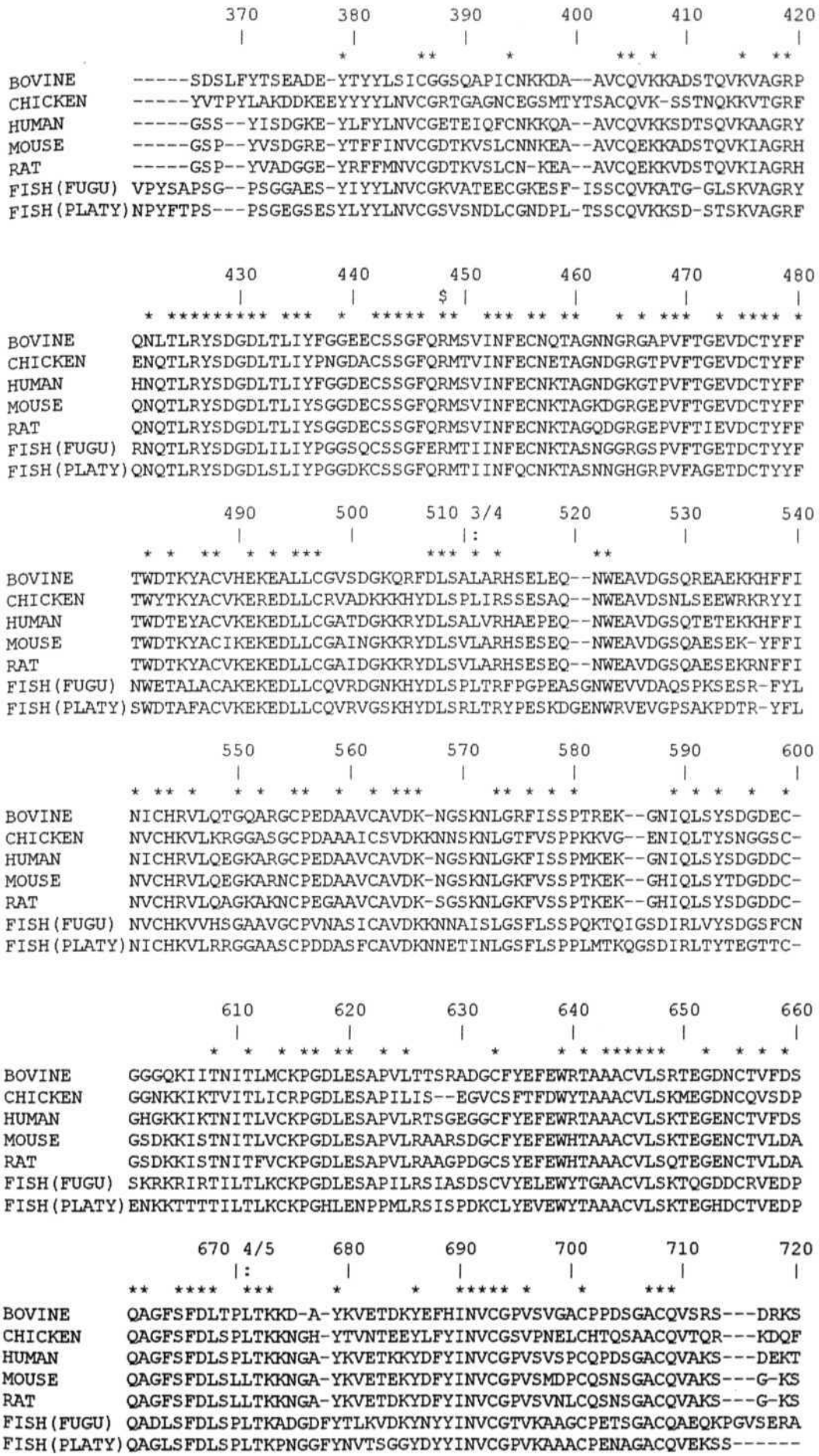
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CHICKEN -----YVTPYLAKDDKEEYYYYLNVCGRTGAGNCEGSMTYTSACQVK-SSTNQKKVTGRF
HUMAN -----GSS--YISDGKE-YLFYLNVCGETEIQFCNKKQA--AVCQVKKSDTSQVKAAGRY
MOUSE -----GSP--YVSDGRE-YTFFINVCGDTKVSLCNNKEA--AVCQEKKADSTQVKIAGRH
RAT -----GSP--YVADGGE-YRFFMNVCGDTKVSLCN-KEA--AVCQEKKVDSTQVKIAGRH
FISH(FUGU) VPYSAPSG--PSGGAES-YIYYLNVCGKVATEECGKESF-ISSCQVKATG-GLSKVAGRY
FISH(PLATY)NPYFTPS---PSGEGSESYLYYLNVCGSVSNDLCGNDPL-TSSCQVKKSD-STSKVAGRF
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CHICKEN ENQTLRYSDGDLTLIYPNGDACSSGFQRMTVINFECNETAGNDGRGTPVFTGEVDCTYFF
HUMAN HNQTLRYSDGDLTLIYFGGDECSSGFQRMSVINFECNKTAGNDGKGTPVFTGEVDCTYFF
MOUSE QNQTLRYSDGDLTLIYSGGDECSSGFQRMSVINFECNKTAGKDGRGEPVFTGEVDCTYFF
RAT QNQTLRYSDGDLTLIYSGGDECSSGFQRMSVINFECNKTAGQDGRGEPVFTIEVDCTYFF
FISH(FUGU) RNQTLRYSDGDLILIYPGGSQCSSGFERMTIINFECNKTASNGGRGSPVFTGETDCTYYF
FISH(PLATY)QNQTLRYSDGDLSLIYPGGDKCSSGFQRMTIINFQCNKTASNNGHGRPVFAGETDCTYYF
490 500 510 3/4 520 530 540
BOVINE TWDTKYACVHEKEALLCGVSDGKQRFDLSALARHSELEQ--NWEAVDGSQREAEKKHFFI
CHICKEN TWYTKYACVKEREDLLCRVADKKKHYDLSPLIRSSESAQ--NWEAVDSNLSEEWRKRYYI
HUMAN TWDTEYACVKEKEDLLCGATDGKKRYDLSALVRHAEPEQ--NWEAVDGSQTETEKKHFFI
MOUSE TWDTKYACIKEKEDLLCGAINGKKRYDLSVLARHSESEQ--NWEAVDGSQAESEK-YFFI
RAT TWDTKYACVKEKEDLLCGAIDGKKRYDLSVLARHSESEQ--NWEAVDGSQAESEKRNFFI
FISH(FUGU) NWETALACAKEKEDLLCQVRDGNKHYDLSPLTRFPGPEASGNWEVVDAQSPKSESR-FYL
FISH(PLATY)SWDTAFACVKEKEDLLCQVRVGSKHYDLSRLTRYPESKDGENWRVEVGPSAKPDTR-YFL
550 560 570 580 590 600
BOVINE NICHRVLQTGQARGCPEDAAVCAVDK-NGSKNLGRFISSPTREK--GNIQLSYSDGDEC-
CHICKEN NVCHKVLKRGGASGCPDAAAICSVDKKNNSKNLGTFVSPPKKVG--ENIQLTYSNGGSC-
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RAT NVCHRVLQAGKAKNCPEGAAVCAVDK-SGSKNLGKFVSSPTKEK--GHIQLSYSDGDDC-
FISH(FUGU) NVCHKVVHSGAAVGCPVNASICAVDKKNNAISLGSFLSSPQKTQIGSDIRLVYSDGSFCN
FISH(PLATY)NICHKVLRRGGAASCPDDASFCAVDKNNETINLGSFLSPPLMTKQGSDIRLTYTEGTTC-
610 620 630 640 650 660
BOVINE GGGQKIITNITLMCKPGDLESAPVLTTSRADGCFYEFEWRTAAACVLSRTEGDNCTVFDS
CHICKEN GGNKKIKTVITLICRPGDLESAPILIS--EGVCSFTFDWYTAAACVLSKMEGDNCQVSDP
HUMAN GHGKKIKTNITLVCKPGDLESAPVLRTSGEGGCFYEFEWRTAAACVLSKTEGENCTVFDS
MOUSE GSDKKISTNITLVCKPGDLESAPVLRAARSDGCFYEFEWHTAAACVLSKTEGENCTVLDA
RAT GSDKKISTNITFVCKPGDLESAPVLRAAGPDGCSYEFEWHTAAACVLSQTEGENCTVLDA
FISH(FUGU) SKRKRIRTILTLKCKPGDLESAPILRSIASDSCVYELEWYTGAACVLSKTQGDDCRVEDP
FISH(PLATY)ENKKTTTTILTLKCKPGHLENPPMLRSISPDKCLYEVEWYTAAACVLSKTEGHDCTVEDP
670 4/5 680 690 700 710 720
BOVINE QAGFSFDLTPLTKKD-A-YKVETDKYEFHINVCGPVSVGACPPDSGACQVSRS---DRKS
CHICKEN QAGFSFDLSPLTKKNGH-YTVNTEEYLFYINVCGSVPNELCHTQSAACQVTQR---KDQF
HUMAN QAGFSFDLSPLTKKNGA-YKVETKKYDFYINVCGPVSVSPCQPDSGACQVAKS---DEKT
MOUSE QAGFSFDLSLLTKKNGA-YKVETEKYDFYINVCGPVSMDPCQSNSGACQVAKS---G-KS
RAT QAGFSFDLSLLTKKNGA-YKVETDKYDFYINVCGPVSVNLCQSNSGACQVAKS---G-KS
FISH(FUGU) QADLSFDLSPLTKADGDFYTLKVDKYNYYINVCGTVKAAGCPETSGACQAEQKPGVSERA
FISH(PLATY)QAGLSFDLSPLTKPNGGFYNVTSGGYDYYINVCGPVKAAACPENAGACQVEKSS------

```
                    730         740         750         760         770         780
                     |           |           |           |           |           |
                  * **     * **** *** *  *  **    * * **  ****  ** * ** * **
BOVINE          WNLGRSNAKLSYYDGMIQLTYRDGTPYNNEKRTPRATLITFLCDRDAGVGFPEYQEEDNS
CHICKEN         WSLGLPNSRLSYYDGLIQLTYKNGTAYNNEKKTQRSTLITFLCDRQAGIGQPEYQVEDNY
HUMAN           WNLGLSNAKLSYYDGMIQLNYRGGTPYNNERHTPRATLITFLCDRDAGVGFPEYQEEDNS
MOUSE           WNLGLSSTKLTYYDGMIQLSYRNGTPYNNEKHTPRATLITFLCDRDAGVGFPEYQEEDNS
RAT             WNLGLSNTKLTYYDGMIQLSYRNGTLYNNEKHTPRSTLITFLCDRDAGVGFPEYQEEDNS
FISH(FUGU)      WSLGQANARLSYYDGLIQLVYSNGSQYNDDRHTARSTLVSFLCDPDAGAGHPEFQVEDSR
FISH(PLATY)     WSLGEANSRLSYYDGLIQLTYSKGSQYNNALHTLRSTLISFLCDPEAGAGSPEFQVEDNY

                    790         800         810  5/6  820         830         840
                     |           |           |    :    |           |           |
                  **** *** ****  * ** ****    ***** *         **             *
BOVINE          TYNFRWYTSYACPEEPLECIVTDPVTLDQYDLSRLAKSEGGPGGNWYSLDNGGARSTWRK
CHICKEN         TYNFRWYTEYACPEMPLECIVTDPNTMDQYDLSSLAKSEK-RGENWYAMDNSGPN-ERKK
HUMAN           TYNFRWYTSYACPEEPLECVVTDPSTLEQYDLSSLAKSEGGLGGNWYAMDNSGEHVTWRK
MOUSE           TYNFRWYTSYACPEEPLECMVTDPSMMEQYDLSSLVKSEGGSGGNWYAMENSREHVTRRK
RAT             TYNFRWYTSYACPEEPLECMVTDPSMMEQYDLSSLVKFEGGRGGNWYAMENSREHFTRRK
FISH(FUGU)      TYNFHWYTSYACPVRPHECLVTDPVTLEQYDLSSLSHSTSA--NNWQVMDYS-DPLNPKK
FISH(PLATY)     TYNFRWYTSYACPQRPHECLVTDPITLDQYDLSSLSRSTSA--NNWVAMDMT-NVSNQKK

                    850         860         870         880         890         900
                     |           |           |           |           |           |
                  ** * ***   * **   ** **  *     *  *    *  *        *     **
BOVINE          YYINVCRPLNPVPGCDRYASACQMKYQGEQGSYSETVSISNLGVAKTGPMVEDSGSLLLE
CHICKEN         YYINVCRPLLAVPGCDRRASVCQMEYRHDHDSYYEVTSISNLGVASKELVVERLGHILLT
HUMAN           YYINVCRPLNPVPGCNRYASACQMKYEKDQGSFTEVVSISNLGMAKTGPVVEDSGSLLLE
MOUSE           YYLNVCRPLNPVPGCDRYASACQMKYENHEGSLAETVSISNLGVAKIGPVVEESGSLLLE
RAT             YYLNVCRPLNPVPGCDRYASACQSKYENNEGSLAETVAISNLGVAKTGPVVEESGSLLLE
FISH(FUGU)      YYFNICRPMNPVLGCDRHASVCQMKYEQE--SLKEVVSVSNMGVAKRGPIIEDRDRLLLE
FISH(PLATY)     YYINVCRPIVPVPGCDHDASVCQMKYITEKGSPKEVVSVSSMGKSKKGPIIEARDRLLLE

                    910         920         930         940         950         960
                     |           |           |           |           |           |
                   ** *         * * **  *  *     * *     *   * * * ***
BOVINE          YVNGSACTTSDQRRTTYTTRIHLVCSTGSLYTHPIFSLNWECVVSFLWNTAAACPIRITT
CHICKEN         YANGSVCINADGERTSYTTTIHFVCSRGTLNSSPRFISIQECVVTFLWETEAACPIKETK
HUMAN           YVNGSACTTSDGRQTTYTTRIHLVCSRGRLNSHPIFSLNWECVVSFLWNTEAACPIQTTT
MOUSE           YVNGSACTTSDGQLTTYSTRIHLVCGRGFMNSHPIFTFNWECVVSFLWNTEAACPIQTIT
RAT             YVNGSACTTSDGRLTTYSTRIHLVCGRGTMNSHPIFTFNWECVVSFLWNTEAACPIQTIT
FISH(FUGU)      FTDGSVCM-SDGQNLSYSTLIHLSCSRGAQSRKPHFMMYQNCTASFMWETRAACAVTTTN
FISH(PLATY)     FTDGSVCM-SEGPRA

                    970         980 6/7     990         1000        1010        1020
                     |           | :         |           |           |           |
                   * *   **  *  * *  *      *       **    * *     **          **
BOVINE          DIDQVCSIKDPNSGYVFDLNPLN-NSRGYVVLGIGKTFLFNVCGDMPACGTLDGKPASGC
CHICKEN         DDSQSCSVRDPNSGFLFNLQPLA-AEKGYTTTGIGKTYLLNICEAMPECGKINGKPAAGC
HUMAN           DTDQACSIRDPNSGFVFNLNPLN-SSQGYNVSGIGKIFMFNVCGTMPVCGTILGKPASGC
MOUSE           ETDQACSIRDPSSGFVFNLSPLNDSAQGHVVLGIGKTFVFNICGAMPACGTVAGKPAYGC
RAT             DSDQACSIRDPNSGFVFNLSPLNYS-QGHMVLGIGKTFVFNICGTMPACGTVAGKPALGC
FISH(FUGU)      S--QNCAVVDPNTGFEFNLQLLA-SENGYQKTANGKVFLVNICSDVKKCGAGI----AGC

                   1030        1040        1050        1060        1070        1080
                     |           |           |           |           |           |
                               *     *  *  *  ** * *                  * *
BOVINE          EAEVQMDDMKTLKPGRLVGLEKSLQLSTEGFITLNYTGLPSHPNGRA--DAFIIRFVCND
CHICKEN         EAD-------NLTSVRMVELDKTLYLSSEGFLTLTYRGPLLVESGKS--DTFTVTFICND
HUMAN           EAETQTEELKNWKPARPVGIEKSLQLSTEGFITLTYKG-PLSAKGTA--DAFIVRFVCND
MOUSE           EAETQIEDIKDLRPQRPVGMERSLQLSAEGFLTLTYKG-S-SPSDRG--TAFIIRFICND
RAT             EAETKIKDIKDLKPERPVGMEKSLQLSAEGFLTLTYKG-S-SPSDRG--TAFIIRFICNG
FISH(FUGU)      V-------LEDGVPVSQVGIEKSLQYSTNGLLTLKYKG-KLD-KPTAKRDTSTINFVCDP
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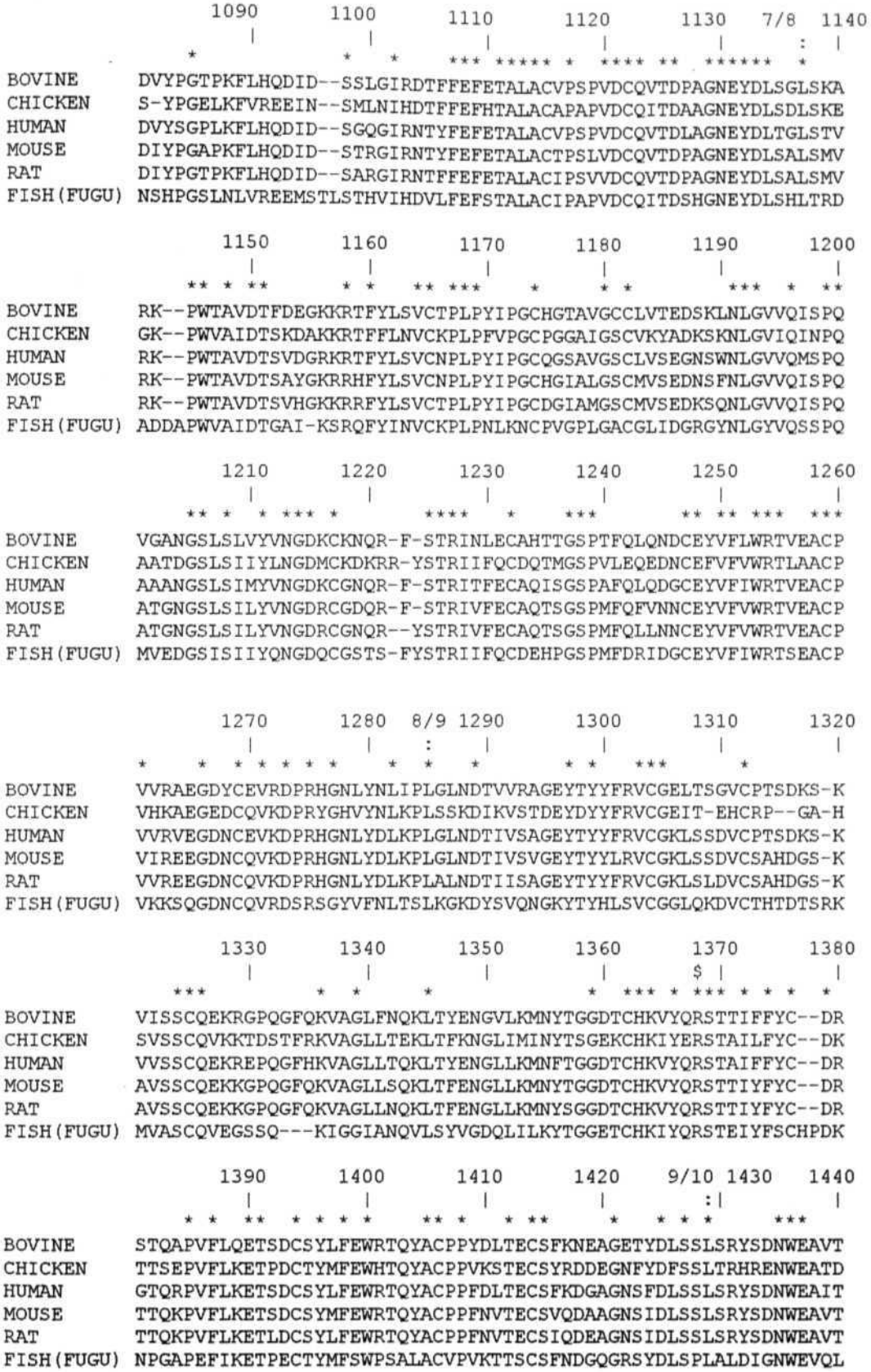
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HUMAN DVYSGPLKFLHQDID--SGQGIRNTYFEFETALACVPSPVDCQVTDLAGNEYDLTGLSTV
MOUSE DIYPGAPKFLHQDID--STRGIRNTYFEFETALACTPSLVDCQVTDPAGNEYDLSALSMV
RAT DIYPGTPKFLHQDID--SARGIRNTFFEFETALACIPSVVDCQVTDPAGNEYDLSALSMV
FISH(FUGU) NSHPGSLNLVREEMSTLSTHVIHDVLFEFSTALACIPAPVDCQITDSHGNEYDLSHLTRD
1150 1160 1170 1180 1190 1200
BOVINE RK--PWTAVDTFDEGKKRTFYLSVCTPLPYIPGCHGTAVGCCLVTEDSKLNLGVVQISPQ
CHICKEN GK--PWVAIDTSKDAKKRTFFLNVCKPLPFVPGCPGGAIGSCVKYADKSKNLGVIQINPQ
HUMAN RK--PWTAVDTSVDGRKRTFYLSVCNPLPYIPGCQGSAVGSCLVSEGNSWNLGVVQMSPQ
MOUSE RK--PWTAVDTSAYGKRRHFYLSVCNPLPYIPGCHGIALGSCMVSEDNSFNLGVVQISPQ
RAT RK--PWTAVDTSVHGKKRRFYLSVCTPLPYIPGCDGIAMGSCMVSEDKSQNLGVVQISPQ
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1210 1220 1230 1240 1250 1260
BOVINE VGANGSLSLVYVNGDKCKNQR-F-STRINLECAHTTGSPTFQLQNDCEYVFLWRTVEACP
CHICKEN AATDGSLSIIYLNGDMCKDKRR-YSTRIIFQCDQTMGSPVLEQEDNCEFVFVWRTLAACP
HUMAN AAANGSLSIMYVNGDKCGNQR-F-STRITFECAQISGSPAFQLQDGCEYVFIWRTVEACP
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RAT ATGNGSLSILYVNGDRCGNQR--YSTRIVFECAQTSGSPMFQLLNNCEYVFVWRTVEACP
FISH(FUGU) MVEDGSISIIYQNGDQCGSTS-FYSTRIIFQCDEHPGSPMFDRIDGCEYVFIWRTSEACP
1270 1280 8/9 1290 1300 1310 1320
BOVINE VVRAEGDYCEVRDPRHGNLYNLIPLGLNDTVVRAGEYTYYFRVCGELTSGVCPTSDKS-K
CHICKEN VHKAEGEDCQVKDPRYGHVYNLKPLSSKDIKVSTDEYDYYFRVCGEIT-EHCRP--GA-H
HUMAN VVRVEGDNCEVKDPRHGNLYDLKPLGLNDTIVSAGEYTYYFRVCGKLSSDVCPTSDKS-K
MOUSE VIREEGDNCQVKDPRHGNLYDLKPLGLNDTIVSVGEYTYYLRVCGKLSSDVCSAHDGS-K
RAT VVREEGDNCQVKDPRHGNLYDLKPLALNDTIISAGEYTYYFRVCGKLSLDVCSAHDGS-K
FISH(FUGU) VKKSQGDNCQVRDSRSGYVFNLTSLKGKDYSVQNGKYTYHLSVCGGLQKDVCTHTDTSRK
1330 1340 1350 1360 1370 1380
BOVINE VISSCQEKRGPQGFQKVAGLFNQKLTYENGVLKMNYTGGDTCHKVYQRSTTIFFYC--DR
CHICKEN SVSSCQVKKTDSTFRKVAGLLTEKLTFKNGLIMINYTSGEKCHKIYERSTAILFYC--DK
HUMAN VVSSCQEKREPQGFHKVAGLLTQKLTYENGLLKMNFTGGDTCHKVYQRSTAIFFYC--DR
MOUSE AVSSCQEKKGPQGFQKVAGLLSQKLTFENGLLKMNYTGGDTCHKVYQRSTTIYFYC--DR
RAT AVSSCQEKKGPQGFQKVAGLLNQKLTFENGLLKMNYSGGDTCHKVYQRSTTIYFYC--DR
FISH(FUGU) MVASCQVEGSSQ---KIGGIANQVLSYVGDQLILKYTGGETCHKIYQRSTEIYFSCHPDK
1390 1400 1410 1420 9/10 1430 1440
BOVINE STQAPVFLQETSDCSYLFEWRTQYACPPYDLTECSFKNEAGETYDLSSLSRYSDNWEAVT
CHICKEN TTSEPVFLKETPDCTYMFEWHTQYACPPVKSTECSYRDDEGNFYDFSSLTRHRENWEATD
HUMAN GTQRPVFLKETSDCSYLFEWRTQYACPPFDLTECSFKDGAGNSFDLSSLSRYSDNWEAIT
MOUSE TTQKPVFLKETSDCSYMFEWRTQYACPPFNVTECSVQDAAGNSIDLSSLSRYSDNWEAVT
RAT TTQKPVFLKETLDCSYLFEWRTQYACPPFNVTECSIQDEAGNSIDLSSLSRYSDNWEAVT
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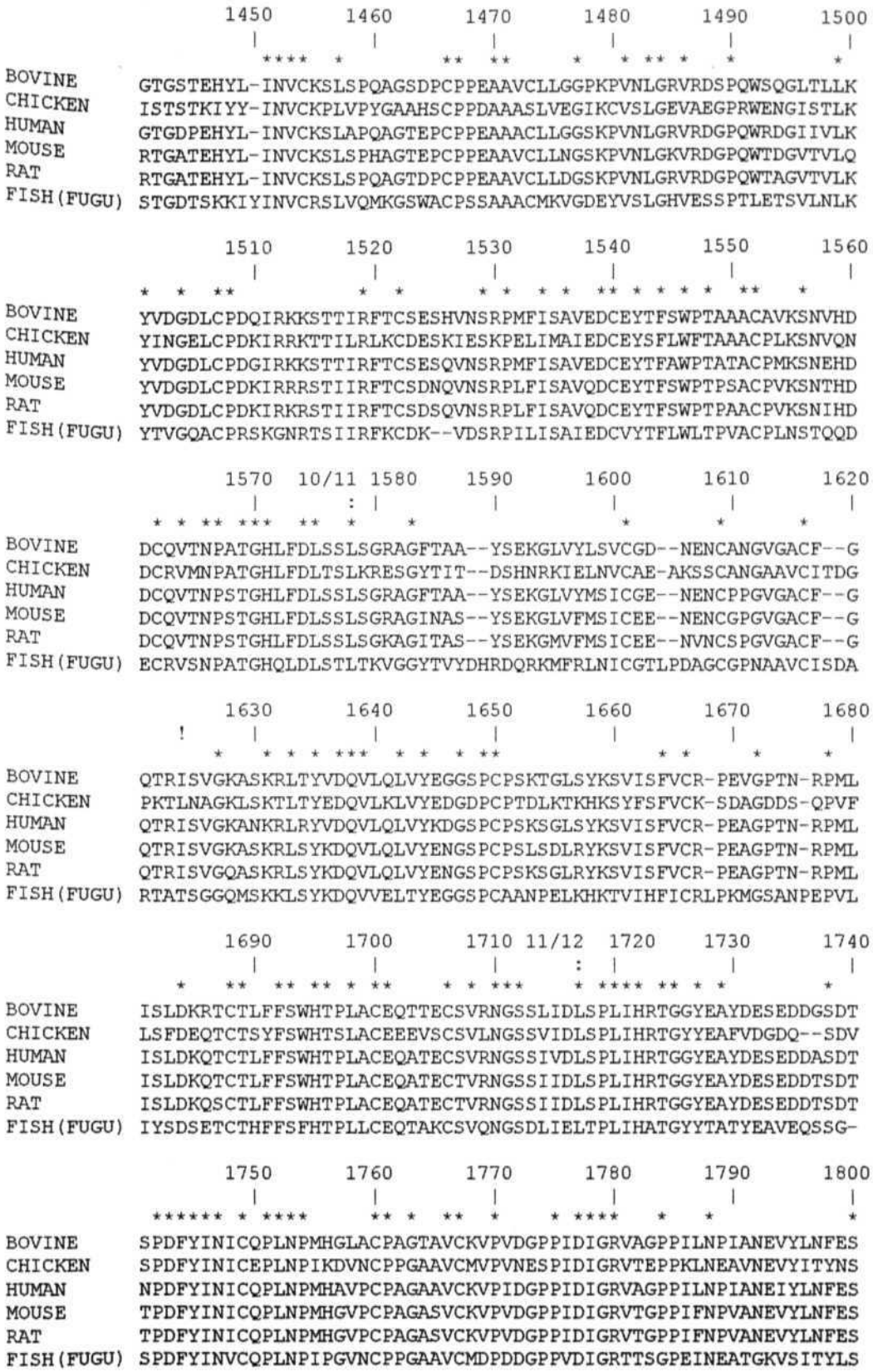
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MOUSE RTGATEHYL-INVCKSLSPHAGTEPCPPEAAVCLLNGSKPVNLGKVRDGPQWTDGVTVLQ
RAT RTGATEHYL-INVCKSLSPQAGTDPCPPEAAVCLLDGSKPVNLGRVRDGPQWTAGVTVLK
FISH(FUGU) STGDTSKKIYINVCRSLVQMKGSWACPSSAAACMKVGDEYVSLGHVESSPTLETSVLNLK
1510 1520 1530 1540 1550 1560
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CHICKEN YINGELCPDKIRRKTTILRLKCDESKIESKPELIMAIEDCEYSFLWFTAAACPLKSNVQN
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MOUSE YVDGDLCPDKIRRRSTIIRFTCSDNQVNSRPLFISAVQDCEYTFSWPTPSACPVKSNTHD
RAT YVDGDLCPDKIRKRSTIIRFTCSDSQVNSRPLFISAVQDCEYTFSWPTPAACPVKSNIHD
FISH(FUGU) YTVGQACPRSKGNRTSIIRFKCDK--VDSRPILISAIEDCVYTFLWLTPVACPLNSTQQD
1570 10/11 1580 1590 1600 1610 1620
BOVINE DCQVTNPATGHLFDLSSLSGRAGFTAA--YSEKGLVYLSVCGD--NENCANGVGACF--G
CHICKEN DCRVMNPATGHLFDLTSLKRESGYTIT--DSHNRKIELNVCAE-AKSSCANGAAVCITDG
HUMAN DCQVTNPSTGHLFDLSSLSGRAGFTAA--YSEKGLVYMSICGE--NENCPPGVGACF--G
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RAT DCQVTNPSTGHLFDLSSLSGKAGITAS--YSEKGMVFMSICEE--NVNCSPGVGACF--G
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BOVINE SPDFYINICQPLNPMHGLACPAGTAVCKVPVDGPPIDIGRVAGPPILNPIANEVYLNFES
CHICKEN SPDFYINICEPLNPIKDVNCPPGAAVCMVPVNESPIDIGRVTEPPKLNEAVNEVYITYNS
HUMAN NPDFYINICQPLNPMHAVPCPAGAAVCKVPIDGPPIDIGRVAGPPILNPIANEIYLNFES
MOUSE TPDFYINICQPLNPMHGVPCPAGASVCKVPVDGPPIDIGRVTGPPIFNPVANEVYLNFES
RAT TPDFYINICQPLNPMHGVPCPAGASVCKVPVDGPPIDIGRVTGPPIFNPVANEVYLNFES
FISH(FUGU) SPDFYINVCQPLNPIPGVNCPPGAAVCMDPDDGPPVDIGRTTSGPEINEATGKVSITYLS

```
                1810       1820       1830       1840       1850       1860
                  |          |          |          |          |          |
              * *      **** * * *  *   * *      *   *** ** ** *     **
BOVINE        STPCLADRHFNYTSLITFHCKRGVSMGTPKLLRTSVCDFVFEWETPLVCPDEVKTDGCSL
CHICKEN       TTPCQINNKLNYTSLIVFHCSQGTSLGKPKMIQKLDCSFVFEWETPVVCPDRVKTLGCSV
HUMAN         STPCLADKHFNYTSLIAFHCKRGVSMGTPKLLRTSECDFVFEWETPVVCPDEVRMDGCTL
MOUSE         STHCLADRYMNYTSLITFHCKRGVSMGTPKLIRTNDCDFVFEWETPIVCPDEVKTQGCAV
RAT           STPCLADKYMNYTSLIAFHCRRGISMGTPKLIRNNDCDFVFEWETPIVCPDEVKTQGCAV
FISH(FUGU)    YTKCAADPSRNYTSTIIFTCQRGLELGSPQMLRLQGCVYLFEWATPIVCSDATSMSGCQL

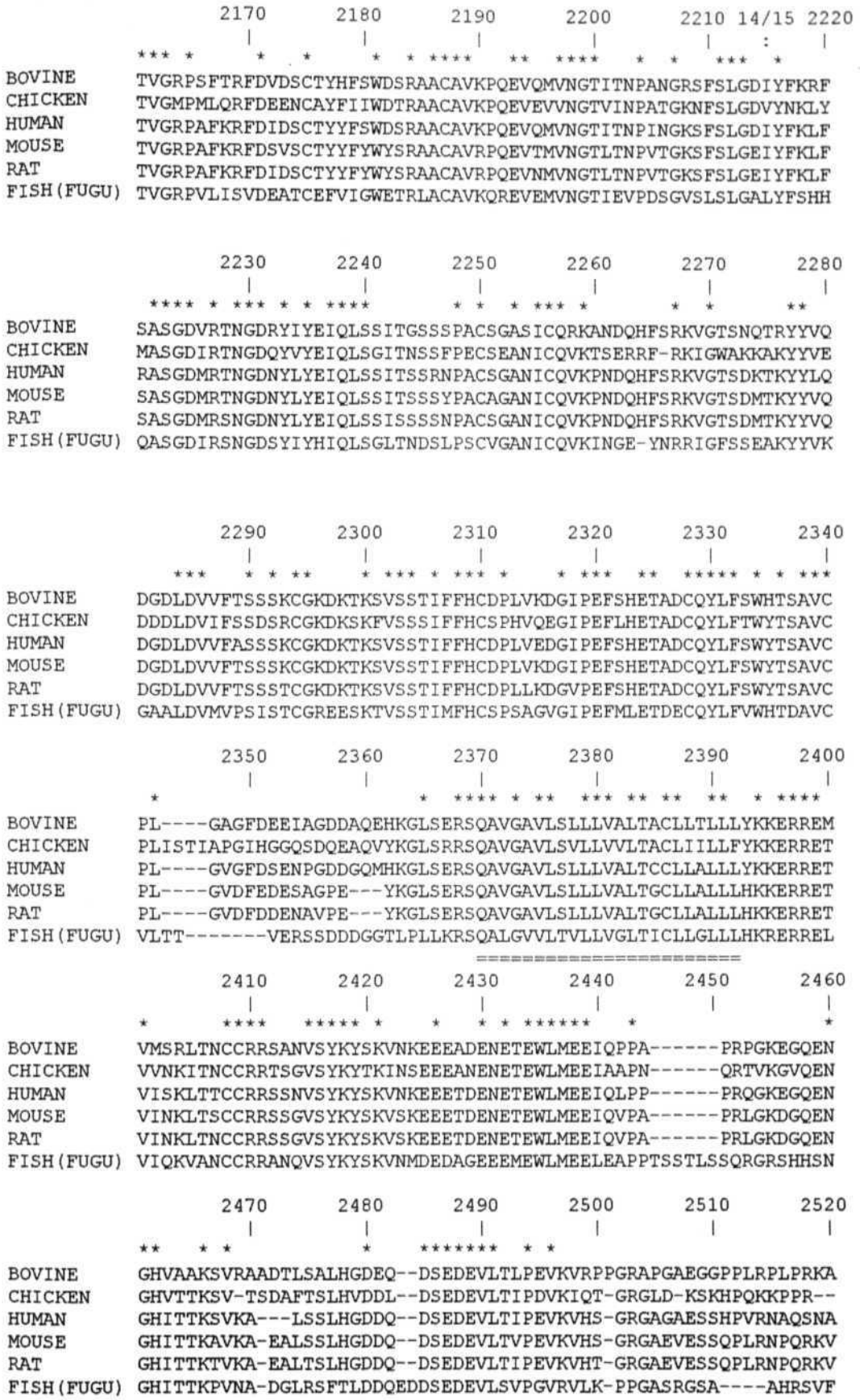
                1870 12/13 1880       1890       1900       1910       1920
                  |   :    |          |          |          |          |
               * **   * *  **     *      *   **          *    **  **
BOVINE        TDEQLYYSFNLSSLSKSTFKVTRGPHTYSVGVCT-AAAGLDEGGCKDGAVCLLSGS----
CHICKEN       TDEQLHYTFNLTSLSGRSFEVLSGSSRYHVSVCSKAAADVSQGKCKDGAVCMTSGS----
HUMAN         TDEQLLYSFNLSSLSTSTFKVTRDSRTYSVGVCT-FAVGPEQGGCKDGGVCLLSGT----
MOUSE         TDEQLLYSFNLTSLSTSTFKVTRDARTYSIGVCT-AAAGLGQEGCKDGGVCLLSGN----
RAT           TDEQLLYSFNLTSLSTSTFKVTRDAHTYSIGVCT-TAADLDQEGCKDGGVCLLSGS----
FISH(FUGU)    NDSQLQFSFDLSVLS-GDVQVPTSSGTYHINVCG-SVTEP---SCENSAVCRVSGSGPEK

                1930       1940       1950       1960       1970       1980
                  |          |          |          |          |          |
             * *    *              *  ** * * * *  * ** **      * *
BOVINE        KGASFGRLASMKLDYRHQDEAVILSYANGDTCPPETEDGEPCVFPFVFNGKSYEECVVES
CHICKEN       GVSSFGSIKEMKMNYSRQDETVILQYTGGDRCPPVTEKGELCVFPFKYKGKSYDKCITEE
HUMAN         KGASFGRLQSMKLDYRHQDEAVVLSYVNGDRCPPETDDGVPCVFPFIFNGKSYEECIIES
MOUSE         KGASFGRLASMQLDYRHQDEAVILSYVNGDPCPPETDDGEPCVFPFIYKGKSYDECVLEG
RAT           KGASFGRLASMQLDYRHQDEAVILSYVNGDPCPPETEDGEPCVFPFIYKGETYDECVLEG
FISH(FUGU)    MVSSYGISKVMTMDFKHDNQGILMEYREGDACSPMTSDGEVCVLPFTFMKKLYKECTKDG

                1990       2000       2010       2020       2030       2040
                  |          |          |          |          |          |
               **  *  *  *  ****      * * * * **     * *   *   **  **
BOVINE        RA--RLWCATTANYDRDHEWGFCKHSTSHRTSVIIFKCDEDADVGRPQVFSEVRGCEVTF
CHICKEN       KN--RPWCATTVDYQGDGKWGFCVNATARRASTIIFVCDENAGSGSPRLLSDMLGCAVTF
HUMAN         RA--KLWCSTTADYDRDHEWGFCRHSNSYRTSSIIFKCDEDEDIGRPQVFSEVRGCDVTF
MOUSE         RA--KLWCSKTANYDRDHEWGFCRQTNSYRMSAIIFTCDESEDIGRPQVFSEDRGCEVTF
RAT           RA--KLWCSKTANYDRDHEWGFCRPSNSHRMSAIIFTCDENEDIGRPEVFSEDRGCEVTF
FISH(FUGU)    RTEGRKWCATTASYDTDGKWGFCTEASAKRRSSILFICDYSAGHGTPQLLSETAGCLATF

                2050       2060       2070 13/14 2080       2090       2100
                  |          |          | :       |          |          |
               ** *  ******  ** *  * * *** *** *  *   * * **  ***     *   *
BOVINE        EWKTKVVCPPKKMECKFVQKHRTYDLRLLSSLTGSWSFVHNGASYYINLCQKIYKGPQDC
CHICKEN       EWRTQAVCPPKKMECKFVQKHRTYDLRILSSLTGSWVFYHNGNSYYLNLCQRVYEGPTGC
HUMAN         EWKTKVVCPPKKLECKFVQKHKTYDLRLLSSLTGSWSLVHNGVSYYINLCQKIYKGPLGC
MOUSE         EWKTKVVCPPKKMECKFVQKHKTYDLRLLSSLTGSWDFVHEGNSYFINLCQRVYKGPLDC
RAT           EWKTKVVCPPKKMECKFVQKHKTYDLRLLSSLTGSWDFVHEGNSYFINLCQRVYKGPLDC
FISH(FUGU)    EWRTSVVCPPKKMDCKLVSQHLTFDLRSLSSMTEPWKFGHHGDSYFMNLCKGVHGGSTGC

                2110       2120       2130       2140       2150       2160
                  |          |          |          |          |          |
               * *  *     *   *** * **           *  *   **        * * *
BOVINE        SERASVCKKSTSGEVQVLGLVHTQKLDVVDDRVIVTYSKGHY-CGDNKTASAVIELTCAK
CHICKEN       PERASICRKSNNGDVEVLGLVHTQKLNVTGDTVYISYSGGQE-CGKNKKIMTIIELRCAR
HUMAN         SERASICRRTTTGDVQVLGLVHTQKLGVIGDKVVVTYSKGYP-CGGNKTASSVIELTCTK
MOUSE         SERASICKKSATGQVQVLGLVHTQKLEVIDETVIVTYSKGYP-CGGNKTASSVIELTCAK
RAT           SERASICKKSATGQVQVLGLVHTQKLEVIDETVLVTYSKGHS-CGGNKTASSVIELTCAK
FISH(FUGU)    PEDAAVCRRSAAGKTQVLGRVFTQRMNYNDGDISVNYAAGDDVCGKGVKATTEIRLSCGS
```


2170 2180 2190 2200 2210 14/15 2220
BOVINE TVGRPSFTRFDVDSCTYHFSWDSRAACAVKPQEVQMVNGTITNPANGRSFSLGDIYFKRF
CHICKEN TVGMPMLQRFDEENCAYFIIWDTRAACAVKPQEVEVVNGTVINPATGKNFSLGDVYNKLY
HUMAN TVGRPAFKRFDIDSCTYYFSWDSRAACAVKPQEVQMVNGTITNPINGKSFSLGDIYFKLF
MOUSE TVGRPAFKRFDSVSCTYYFYWYSRAACAVRPQEVTMVNGTLTNPVTGKSFSLGEIYFKLF
RAT TVGRPAFKRFDIDSCTYYFYWYSRAACAVRPQEVNMVNGTLTNPVTGKSFSLGEIYFKLF
FISH(FUGU) TVGRPVLISVDEATCEFVIGWETRLACAVKQREVEMVNGTIEVPDSGVSLSLGALYFSHH
2230 2240 2250 2260 2270 2280
BOVINE SASGDVRTNGDRYIYEIQLSSITGSSSPACSGASICQRKANDQHFSRKVGTSNQTRYYVQ
CHICKEN MASGDIRTNGDQYVYEIQLSGITNSSFPECSEANICQVKTSERRF-RKIGWAKKAKYYVE
HUMAN RASGDMRTNGDNYLYEIQLSSITSSRNPACSGANICQVKPNDQHFSRKVGTSDKTKYYLQ
MOUSE SASGDMRTNGDNYLYEIQLSSITSSSYPACAGANICQVKPNDQHFSRKVGTSDMTKYYVQ
RAT SASGDMRSNGDNYLYEIQLSSISSSSNPACSGANICQVKPNDQHFSRKVGTSDMTKYYVQ
FISH(FUGU) QASGDIRSNGDSYIYHIQLSGLTNDSLPSCVGANICQVKINGE-YNRRIGFSSEAKYYVK
2290 2300 2310 2320 2330 2340
BOVINE DGDLDVVFTSSSKCGKDKTKSVSSTIFFHCDPLVKDGIPEFSHETADCQYLFSWHTSAVC
CHICKEN DDDLDVIFSSDSRCGKDKSKFVSSSIFFHCSPHVQEGIPEFLHETADCQYLFTWYTSAVC
HUMAN DGDLDVVFASSSKCGKDKTKSVSSTIFFHCDPLVEDGIPEFSHETADCQYLFSWYTSAVC
MOUSE DGDLDVVFTSSSKCGKDKTKSVSSTIFFHCDPLVKDGIPEFSHETADCQYLFSWYTSAVC
RAT DGDLDVVFTSSSTCGKDKTKSVSSTIFFHCDPLLKDGVPEFSHETADCQYLFSWYTSAVC
FISH(FUGU) GAALDVMVPSISTCGREESKTVSSTIMFHCSPSAGVGIPEFMLETDECQYLFVWHTDAVC
2350 2360 2370 2380 2390 2400
BOVINE PL----GAGFDEEIAGDDAQEHKGLSERSQAVGAVLSLLLVALTACLLTLLLYKKERREM
CHICKEN PLISTIAPGIHGGQSDQEAQVYKGLSRRSQAVGAVLSVLLVVLTACLIILLFYKKERRET
HUMAN PL----GVGFDSENPGDDGQMHKGLSERSQAVGAVLSLLLVALTCCLLALLLYKKERRET
MOUSE PL----GVDFEDESAGPE---YKGLSERSQAVGAVLSLLLVALTGCLLALLLHKKERRET
RAT PL----GVDFDDENAVPE---YKGLSERSQAVGAVLSLLLVALTGCLLALLLHKKERRET
FISH(FUGU) VLTT-------VERSSDDDGGTLPLLKRSQALGVVLTVLLVGLTICLLGLLLHKRERREL
2410 2420 2430 2440 2450 2460
BOVINE VMSRLTNCCRRSANVSYKYSKVNKEEEADENETEWLMEEIQPPA------PRPGKEGQEN
CHICKEN VVNKITNCCRRTSGVSYKYTKINSEEEANENETEWLMEEIAAPN------QRTVKGVQEN
HUMAN VISKLTTCCRRSSNVSYKYSKVNKEEETDENETEWLMEEIQLPP------PRQGKEGQEN
MOUSE VINKLTSCCRRSSGVSYKYSKVSKEEETDENETEWLMEEIQVPA------PRLGKDGQEN
RAT VINKLTNCCRRSSGVSYKYSKVSKEEETDENETEWLMEEIQVPA------PRLGKDGQEN
FISH(FUGU) VIQKVANCCRRANQVSYKYSKVNMDEDAGEEEMEWLMEELEAPPTSSTLSSQRGRSHHSN
2470 2480 2490 2500 2510 2520
BOVINE GHVAAKSVRAADTLSALHGDEQ--DSEDEVLTLPEVKVRPPGRAPGAEGGPPLRPLPRKA
CHICKEN GHVTTKSV-TSDAFTSLHVDDL--DSEDEVLTIPDVKIQT-GRGLD-KSKHPQKKPPR--
HUMAN GHITTKSVKA---LSSLHGDDQ--DSEDEVLTIPEVKVHS-GRGAGAESSHPVRNAQSNA
MOUSE GHITTKAVKA-EALSSLHGDDQ--DSEDEVLTVPEVKVHS-GRGAEVESSQPLRNPQRKV
RAT GHITTKTVKA-EALTSLHGDDQ--DSEDEVLTIPEVKVHT-GRGAEVESSQPLRNPQRKV
FISH(FUGU) GHITTKPVNA-DGLRSFTLDDQEDDSEDEVLSVPGVRVLK-PPGASRGSA----AHRSVF

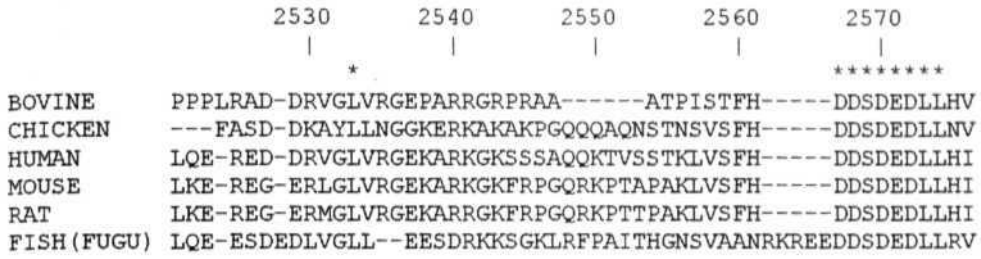


Figure.9 : Alignment of amino acid sequence of Fugu MPR 300 with other vertebrate MPR 300 sequences.

The amino acid numbers will not correspond to those generated for each species separately because gaps(——)were inserted to align the homologous amino acids. The asterisk(*)indicates a perfect amino acid agreement. (===)underlines the predicted transmembrane domains.(. . . .) indicates the acidic cluster found at the C-terminus that contains a casein kinase 2 site and has a function in lysosomal enzyme targeting. The domain borders are marked, the numbers indicate the respective domains, the slash(:)indicates the last amino acid of a domain. ($) marks the conserved arginine residues involved in mannose-6-phosphate binding in domains 3 and 9. (!) marks the residue in domain 11 that is critical for IGF-II binding.

Figure. 10 : Northern blot analysis of fugu MPR 300. Fugu MPR 300 mRNA transcript detected by Northern blot analysis. 10 μg of total RNA isolated from different tissues of fugu was subjected to denaturing agarose gel electrophoresis, transferred to hybond-N nylon membrane and hybridized with ^{32}P labeled fugu MPR 300 1.45 kb cDNA fragment. A single band of 9.5 kb mRNA transcript was obtained in some tissues. Actin was taken as positive control.

Table.5 : Table showing different mRNA transcript lengths coding for MPR 300 from various species as indicated.

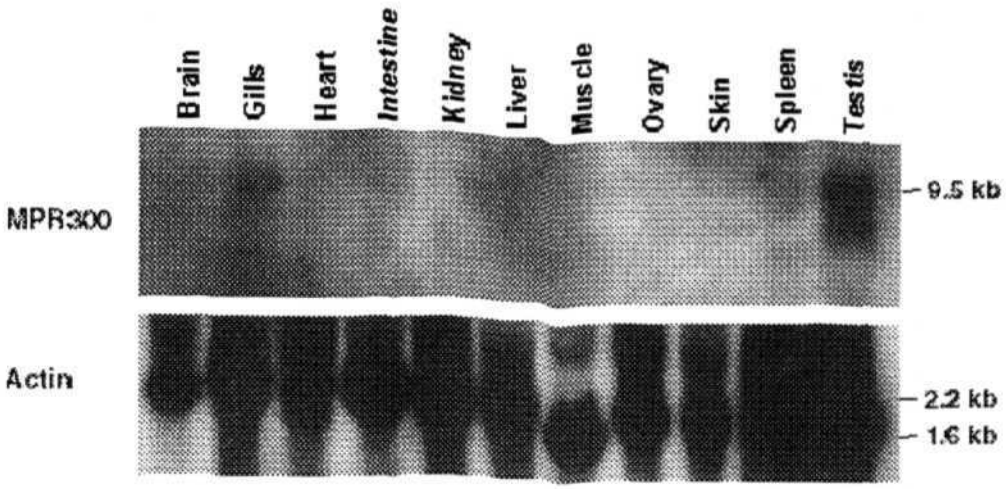


Figure. 10

S.No	Species	mRNA Transcript Length for MPR 300	Coding Sequence Length
1	Human	~ 9.4 kb	7.41 kb
2	Bovine	~ 9.5 kb	7.50 kb
3	Mouse	~ 9-9.5 kb	7.43 kb
4	Chicken	~ 11.0 kb	7.44 kb
5	Fish	~ 9.5 kb	7.49 kb

Table.5

DISCUSSION

MPR proteins have been extensively characterised among the mammals and aves (Hille-Rehfeld, 1995). The first biochemical evidence for the presence of both MPR proteins among other non-mammalian vertebrates such as the reptiles, amphibians and fish came from our studies (Siva Kumar *et al*, 1997; 1999). The luminal domain of MPR 46 exhibits 14-37% similarity to the 15 internal cassettes of MPR 300 as shown for the bovine MPRs (Lobel *et al*, 1988). This finding led to the assumption that MPR 46 is the most ancient in evolution and MPR 300 arouse by multiple gene duplication from MPR 46. A partial cDNA sequence for the fish MPR 300 isolated and characterised in our laboratory showed extensive sequence homology with other known vertebrate MPR 300 proteins (Udaya Lakshmi *et al,* 2000). To further obtain full length clone of the fish MPR 300 xiphophorus cDNA library screening was performed. A 1.25 kb mouse MPR 300 fragment labeled was used as a probe to screen the fish cDNA library. Some clones were identified as positive and the screening was continued till tertiary screening. Two clones from the tertiary screening were sequenced. However, these clones showed homology with ubiquitin but not with MPR 300.

When this work was under progress the complete genome sequence of the fugu fish genome was nearing completion and has recently been published in Science (2002). A thorough search of the genomic data suggested possible sequences of the MPR 300 and MPR 46. Based on the genomic data available primers were designed and initially a 1.45 kb fragment for fugu MPR 300 which spans from domain 8 to 11 was obtained using fugu liver total RNA by RT-PCR approach. This fragment at the protein level shows homology with other MPR 300 protein sequences. Gene specific primers were designed to get the full length sequence of fugu MPR 300.

A 7564 bp cDNA sequence of MPR 300 gene was isolated from Fugu liver total RNA which consists of a 73 bp 5' UTR, 7488 bp ORF and a stop codon. The cDNA sequence encodes for 2496 amino acids, which contains a 33 residue signal peptide, extra cytoplasmic domain in 15 repetitive domains, transmembrane domain and a cytoplasmic domain.

Several lines of evidence suggest that the cDNA clone isolated in the study for fijgu MPR 300 indeed represent the putative receptor. First, the full length cDNA sequence of fugu MPR 300 shows homology with other known MPR 300 proteins. Second, the transmembrane domain is highly conserved among all receptors. Third, the cysteine residues that aid in disulfide pairing in extra cytoplasmic domain of the receptor are highly conserved among all receptors. Fourth, the ligand binding arginine residues in domain 3 and 9 are highly conserved among all the vertebrates. Further, it has 17 N-glycosylation sites.

However, the IGF-II binding site in domain 11 has a threonine residue in fish receptor as compared to the isoleucine in mammalian species. Earlier it was thought that IGF-II binds only to MPR 300 in viviparous animals but, recently there was an article from Mendez *etal*., in which they showed that IGF-II also binds to fish MPR 300. So it remains to be established whether fugu MPR 300 can bind IGF-II.

Northern blot analysis shows a 9.5 kb band corresponding to MPR 300 as compared to other MPR 300 transcript sizes of 9.0-11.5 kb. The differential levels of expression in different tissues are because the MPR is expressed not at same level in all the tissues but it differs from tissue to tissue and also depends on age of the organism. This is further supported by our recent studies where we have shown the levels of both receptors vary in different tissues in the same animal (Suresh, *et al*, 2002).

CHAPTER III

MOLECULAR CLONING AND SEQUENCING OF FISH MPR 46

INTRODUCTION

The mammalian MPR 46 (human, mouse, bovine) sequences show extensive homologies. Among the non-mammalian vertebrates, only a partial sequence of the cytoplasmic tail of chicken MPR 46 has been reported (Matzner *et al,* 1996). Presence of putative MPR 46 in *Xiphophorus* cell lines was established from our laboratory (Siva kumarefa/, 1999).

In a recent study, a partial cDNA clone for the fish MPR 300 that shows sequence similarity to the other known vertebrate MPR 300 proteins has also been reported (Udaya lakshmi *et al,* 2000), suggesting that this protein is conserved throughout vertebrates.

Since already the fish MPR 300 sequence has been obtained in this study, we wanted to isolate and sequence a full length cDNA clone for the fish MPR 46 protein, to further gain insight into the evolution of these interesting proteins.

A RT-PCR approach was used for obtaining the sequence of the fugu MPR 46 and the xiphophorus MPR 46 protein.

MATERIALS

Most of the equipments, chemicals, kits and enzymes used for molecular cloning work have been described in detail in chapter 2.

Pfu DNA polymerase	MBI fermentas
HAMs F12 medium	Sigma
Fetal calf serum	Sigma

METHODS

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.

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 was taken for counting in Neubauer chamber. Cells were counted in 16 squares (4 x 4) at 2 different regions and the average was taken.

Number 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 1.0 mL of PBS. Cells were harvested (2 x 1.0 mL PBS) with a sterile disposable rubber spatula and transferred to a sterile tube and 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 upto 1 year without any degradation.

- Total RNA from the A2 cell pellets was isolated as described in the earlier chapter.

Northern Blotting (RNA Transfer):

Southern, 1975.

The total RNA from different tissues of fugu has been isolated and electrophorosed on agarose gel electrophoresis as described in chapter 2. The bands were transferred to membrane and the membrane was processed as described for MPR 300. A 570 bp RT-PCR product of fugu MPR 46 labeled probe was used for identifying the RNA transcript size of fugu MPR 46.

Long Term Storage of Bacterial Stocks:

Different clones for both MPR 300 and MPR 46 were stored as bacterial stocks as described:

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 were 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.

RESULTS

Amplification of Fugu **MPR** 46 cDNA sequence using **RT-PCR approach**

A thorough data base search of the fugu genome sequences showed that the genome contains possible sequences that are related to the Mannose 6-phosphate receptor proteins (MPR 46 and MPR 300). To get the cDNA sequence of the putative MPR 46 protein, we used the single stranded cDNA of fugu prepared in a PCR experiment employing the following primer pairs (GGTGCTGGAGTGTGATTCAAATAGAT forward and reverse primer TAGTAAGTGGTCATCTTGCTCCTC). A 570 bp fragment **(Fig.** 11 A) was amplified. When this was subcloned into TA cloning vector, and the plasmid DNA isolated and sequenced, the sequence results indicated extensive sequence homologies to the other known MPR proteins (data not shown). In order to get the full length clone of the fugu MPR 46 protein, RACE PCR was done according to manufacturers protocol (CLONTECH) using primers from 570 bp fragment (GTTATCTATTTGAATCACTCCAGC and GTAGGTCAGAAACACCCAGTTAC). This resulted in obtaining additional fragments, which when subcloned and sequenced resulted in obtaining the start codon and the 5'UTR sequences. In order to obtain the entire ORF sequence in one fragment the following primer pairs were used ATGAAGATGGTCAACAGTTATAC and TCACATAGGTAGTAAGTGGTCATC. This resulted in the amplification of a single fragment of 825 bp, **(Fig.** 11 B) which was subcloned into a TA cloning vector. Bacteria were transformed and the positive clones were isolated by blue-white selection. From the positive clones, plasmid DNA was isolated and subjected to EcoRI digestion as described under methods. The results indicate the presence of the right insert.

Figure. 11 : Restriction analysis of plasmid DNA isolated from TA clones of fugu MPR 46 fragments. Plasmid DNA isolated from TA clones was subjected to restriction digestion with EcoR I and fragments were analyzed by 2% agarose gel electrophoresis. Panel A, 570 bp cDNA fragment of fugu MPR 46. Panel B, 825 bp cDNA fragment of fugu MPR 46. The fragments are indicated with arrow.
Lane 1, standard DNA ladder; **Lane 2,** Fugu MPR 46 cDNA fragment;
Lane 3, plasmid DNA digested with EcoR I.

Figure. 12 : Strategy adopted to obtain different cDNA fragments of fugu, MPR 46.
The figure shows different cDNA fragments generated for fugu MPR 46 using the gene specific primer pairs which are indicated above. The arrow indicate the start codon and stop codon of the sequence.

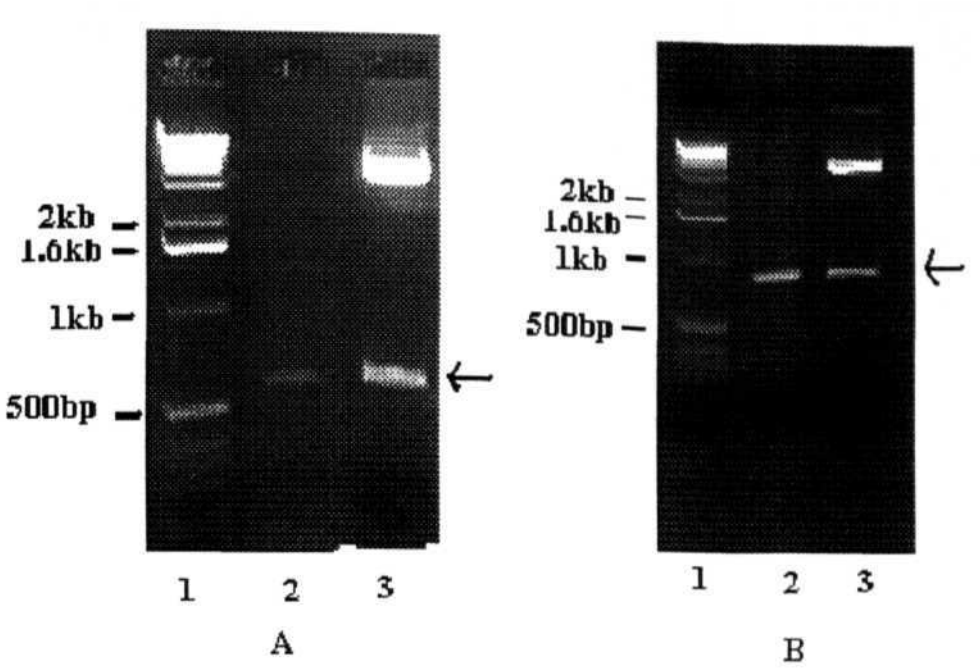


Figure. 11

Primers used for obtaining 570 bp fragment:

sense-5'GGTGCTGGAGTGATTCAAATAGAT3'

antisense-5'TAGTAAGTGGTCATCTCGCTCCTC3'

Primers used for obtaining 825 bp fragment:

sense-5'ATGAAGATGGTCAACAGTTATAC3'

antisense-5'TCACATAGGTAGTAAGTGGTCATC3'

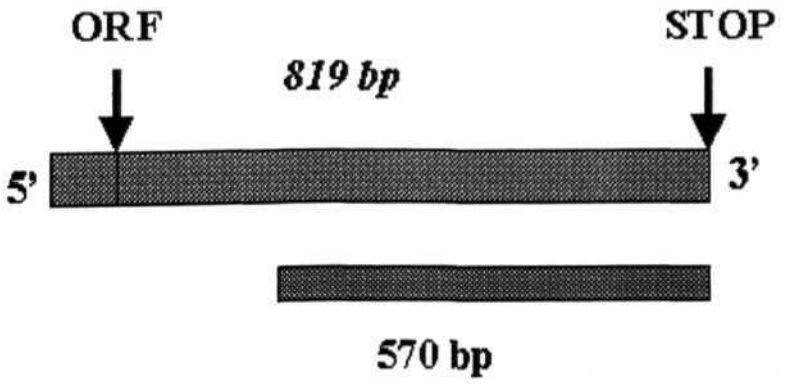


Figure. 12

S.NO	Primer Sense(S)/ Antisense (AS)	Primer	Location Domain	Primer sequence 5' → 3'
1	S	RAJU46-1	AMI TER	GGTGCTGGAGTGATTCAAATAGAT
2	S	RAJU46-2	CYT TAIL	GCTGTGACTTTGTGTGCCGCTTC
3	AS	RAJU46-3	CYT TAIL	TAGTAAGTGGTCATCTCGCTCCTC
4	S	RAJU46-4	AMI TER	AC/TAGAAAAAA/TCT/A/GTGC/TGACC/TTG
5	S	RAJU46-5	AMI TER	GGAGAA/GAAGGG/ATAAA/GGAG/ATCAG/AAG
6	AS	RAJU46-6	AMI TER	GTAGGTCAGAAACACCCAGTTAC
7	AS	RAJU46-7	AMI TER	GTTATCTATTTGAATCACTCCAGC
8	AS	RAJU46-8	CYT TAIL	TCACATAGGTAGTAAGTGGTCATC
9	S	RAJU46-9	5VATG	ATGAAGATGGTCAACAGTTATAC
10		UP SHORT		CTAATACGACTCACTATAGGGC

Table.6 : List of primers used to obtain the sequence of fugu MPR 46.
AMI TER -Amino terminal and CYT TAIL - Cytoplasmic tail

Analysis of fugu MPR 46 sequence data:

From **Fig.** 13 it is apparent that the single fragment of fugu MPR 46 cDNA contains a 81-bp 5'UTR, a 816-bp ORF and a stop codon. The 5'UTR contains an inframe termination codon at nucleotides -28 to -30. The cDNA encodes for a protein of 272 amino acids whose sequences are shown in comparison with other known vertebrate MPR 46 proteins (Fig. 14). The data clearly indicate the presence of a 28 amino acid putative signal sequence, an extracytoplasmic domain, transmembrane domain and a cytoplasmic domain that are characteristics of MPR 46 protein from mammals.

When the DNA was sequenced and the sequences compared to known MPR protein sequences, it showed extensive homology with the known mammalian MPR 46

5'UTR ATCTGAAGGACTGTTGCTGCGTTAGCGATACAGGAG
AAATAGTCATTTTCTTAATCATTTGCAGTGCAAAACATCATGAAG

1 atg gtc aac agt tat act aga ggg tcc ctc ttg atg tgg gct ctg get ctt tgg gtg att

M V N S Y T R G S L L M W A L A L W V I

61 ctg gat gga ggt cca gtg tgc gct aca aac agc acc cag age tgc aaa ctc ttg tct gag

L D G GP VC A AT N S T Q S C K L L S E

121 tea gac cgg aaa gtc ctc agt cag ctt gag cca ctt get cat cag aac ttt tca gga cag

S D R K V L S Q L E P L A H Q N F S G Q

181 age aat aat gag aat tac acg tac gtg ttc cag ctg tgt gga gat gca ggt ggc atc tcc

S N N E N Y T Y V F Q L C G D A GG I S

241 ggt get gga gtg att caa ata gat aac aag aag aag gaa gee aag ccc aca gtg atc gga

G A G V I Q I D N K K K E A K P T V I G

301 aca tat aat tta aca cag gcc att gga gaa agt aac tgg gtg gtt ctg acc tac aca tac

T Y N L T Q Al G E S N W V V L T Y T Y

361 aac aac aaa cat gga tcc tgt tct aaa gat gcg age cag gcc caa atc atg ate ttt tgc

N N K H G S C S K D A S Q A Q I M I F C

421 aac agg gag gta gat gag age cca ctg cat gtg atc cag gag aac agt gag aga gag aac

N R E V D E S P L H V 1 Q E N S E R E N

481 cac tgt ttg tac ctg ttc agg ttg gac tcc age gcc gtg tgc cca get ctt cag tcc aag

H C L Y L F R LD S S AVC P A L Q S K

541 ctc agc gcc ggt tec att ate ctc atc att ggg ttc tgc ctc ctg acg gtt tac ctc gtt

L S A G S 1 1 L I 1 G F C L L T V Y L V

601 gga ggt ttc ctc tac cag aga atg att gtt gga get aaa ggg atg gag cag ttc cca aat

G G F L Y Q R M 1 V G A K G M E Q F P N

661 tac gcc ttc tgc gtg gag gtt ggg aat ctg gca gcg gat ggc tgt gac ttt gtg tgc cgc

Y A F C V E V G N L A AD G C D F V C R

721 tct aga act cgc gag gaa gtc ccc acc tac aga ggc gtt tct tct gaa cct tca gag gac

S R T R E E V P T Y R G V S S E P S E D

781 gat cca gag gag cga gat gac cac tta cta cct atg tga

D P E E R D D H L L P M *

Figure. 13 : Nucleotide and deduced amino acid sequence of full length fugu MPR 46 cDNA.

▲ indicates the signal peptide cleavage site. N glycosylation sites are under lined, * indicates the stop codon.

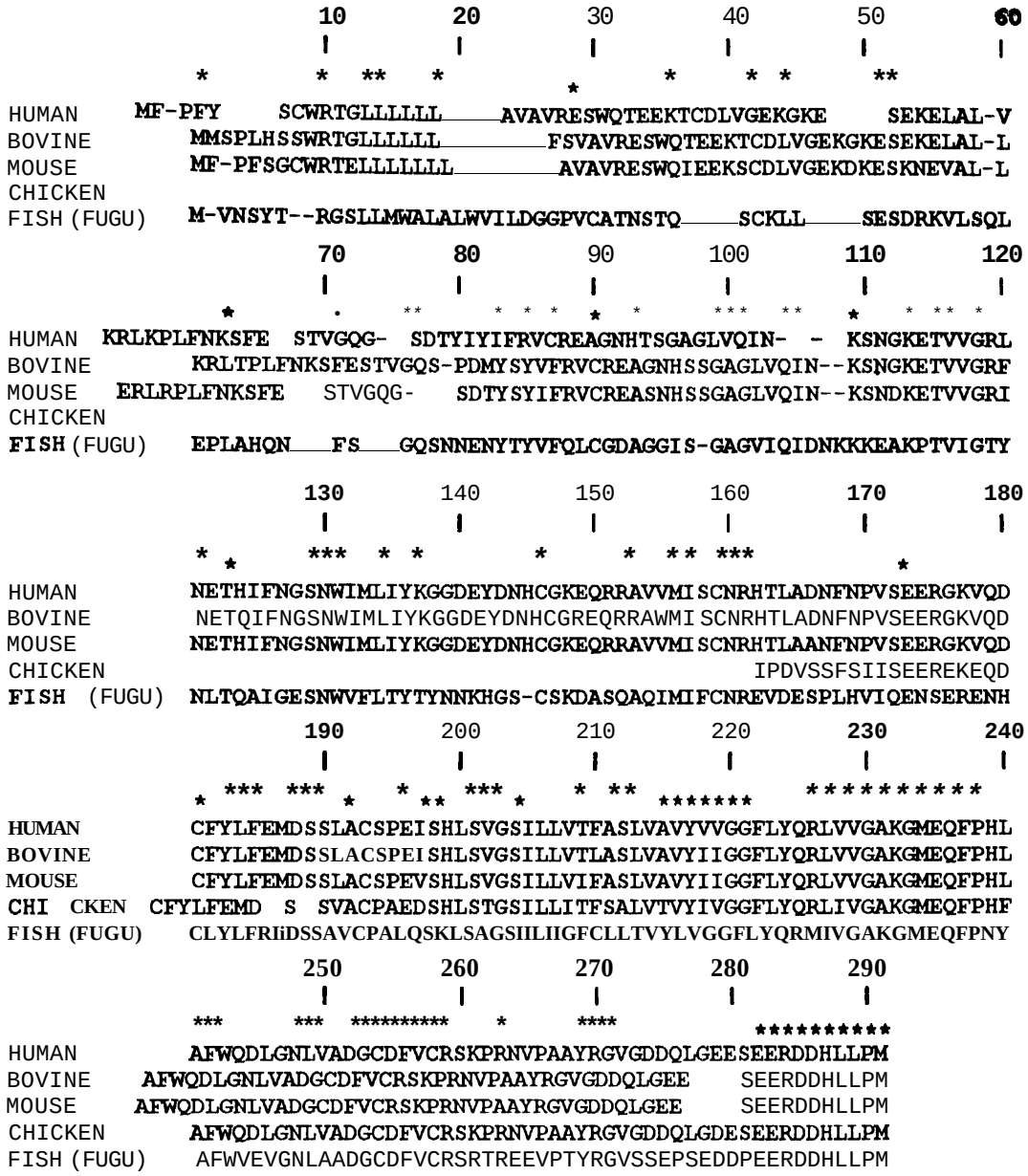


Figure. 14 : Alignment of amino acid sequence of fugu MPR 46 with other vertebrate MPR 46 sequences. The amino acid numbers will not correspond to those generated for each species separately because gaps(——)were inserted to align the homologous amino acids. The asterisk (*)indicates a perfect amino acid agreement.

sequences. Of particular interest are the highly conserved cysteine residues, the transmembrane domain and the cytoplsamic tail. It has 4 potential N-glycosylation sites.

Xiphophorus MPR 46

Since already the putative MPR 46 was identified in fish cell line, *xiphophorus* A2 cells, we wanted to clone this gene using total RNA from fish cells and by employing RT-PCR approach. The primers that worked successfully with fugu were used in this study. Full length sequence was obtained for MPR 46 (Fig. 15) which shows 98% homology with fugu MPR 46 (**Fig.16**).

Northern blot analysis :

For northern blot analysis total RNA was taken from different tissues and hybridised with 570 bp fragment obtained by RT-PCR (this fragment showed extensive sequence homology to the mammalian MPR 46 proteins, data not shown). The results show a band, which coincides with the MPR 46 RNA of other species detected earlier with a size of 2.3 kb (Fig .17). Table 7 summarizes the structural similarity among the known MPR proteins.

5'UTR CTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGC
TCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTATGAAG

1 atg gtc aac agt tat act aga ggg tcc ctc ttg atg tgg gct ctg get ctt tgg gtg att

M V N S Y T R G S L L M W A L A L W V 1

61 ctg gat gga ggt cca gtg tgc gct aca aac agc acc cag agc tgc aaa ctc ttg tct gag

L D G G P V C AAT N S T Q S C K L L S E

121 tca gac egg aaa gtc etc agt cag ctt gag cca ctt gct cat cag aac ttt tca gga cag

S D R K V L S Q L E P L A H Q N F S G Q

181 agc aat aat gag aat tac acg tac gtg ttc cag ctg tgt gga gat gca ggt ggc ate tcc

S N N E N Y T Y V F Q L C G D A G G I S

241 ggt gct gga gtg att caa ata gat aac aag aag aag gaa gcc aag ccc aca gtg atc gga

G A G V I Q I D N K K K E A K P T V I G

301 aca tat aat tta aca cag gcc att gga gaa agt aac tgg gtg ttt ctg acc tac ata tac

T Y N L T Q A 1 G E S N W V F L T Y 1 Y

361 aac aac aaa cat gga tcc tgt tct aaa gat gcg age cgg gcc caa atc atg atc ttt tgc

N N K H G S C S K D A S R A Q I M I F C

421 aac agg gag gta gat gag age cca ctg cat gtg atc cag gag aac agt gag aga gag gac

N R E V D E S P L H V I Q E N S E R E D

481 cac tgt ttg tac ctg ttc agg ttg gac tcc agc gcc gtg tgc cca gct ctt cag tcc aag

H C L Y L F R L D S S A V C P A L Q S K

541 etc age gcc ggt tcc att atc etc ate att ggg ttc tgc etc ctg acg gtt tac etc gtt

L S A G S I I L I I G F C L L T V Y L V

601 gga ggt ttc ctc tac cag aga atg att gtt gga gct aaa ggg atg gag cag ttc cca aat

G G F L Y Q R M I V G A K G M E Q F P N

661 tac gcc ttc tgg gtg gag gtt ggg aat ctg gca gcg gat ggc tgt gac ttt gtg tgc cgc

Y A F W V E V G N L A A D G C D F V C R

721 tct aga act cgc gag gaa gtc ccc acc tac aga ggc gtt tct tct gaa cct tea gag gac

S R T R E E V P T Y R G V S S E P S E D

781 gat cca gag gag cga gat gac cac tta cta cct atg tga

D P E E R D D H L L P M *

Figure. 15 : Nucleotide and deduced amino acid sequence of full length *xiphophorus* MPR 46 cDNA.

▲ indicates the signal peptide cleavage site. N glycosylation sites are under lined. * indicates the stop codon.

```
FUGU          1 MVNSYTRGSLLMWALALWVILDGGPVCATNSTQSCKLLSESDRKVLSQLEPLAHQNFSGQ
XIPHOPHORUS   1 MVNSYTRGSLLMWALALWVILDGGPVCATNSTQSCKLLSESDRKVLSQLEPLAHQNFSGQ
                ************************************************************

FUGU         61 SNNENYTYVFQLCGDAGGISGAGVIQIDNKKKEAKPTVIGTYNLTQAIGESNWVVLTYTY
XIPHOPHORUS  61 SNNENYTYVFQLCGDAGGISGAGVIQIDNKKKEAKPTVIGTYNLTQAIGESNWVFLTYIY
                ************************************************** *** *

FUGU        121 NNKHGSCSKDASQAQIMIFCNREVDESPLHVIQENSERENHCLYLFRLDSSAVCPALQSK
XIPHOPHORUS 121 NNKHGSCSKDASRAQIMIFCNREVDESPLHVIQENSEREDHCLYLFRLDSSAVCPALQSK
                ************ ************************* ********************

FUGU        181 LSAGSIILIIGFCLLTVYLVGGFLYQRMIVGAKGMEQFPNYAFCVEVGNLAADGCDFVCR
XIPHOPHORUS 181 LSAGSIILIIGFCLLTVYLVGGFLYQRMIVGAKGMEQFPNYAFWVEVGNLAADGCDFVCR
                ****************************************** ****************

FUGU        241 SRTREEVPTYRGVSSEPSEDDPEERDDHLLPM
XIPHOPHORUS 241 SRTREEVPTYRGVSSEPSEDDPEERDDHLLPM
                ********************************
```

Figure.16 : Alignment of amino acid sequence **of fugu MPR 46 with *xiphophorus* MPR 46** sequence. Conserved amino acids are indicated with asterisks.

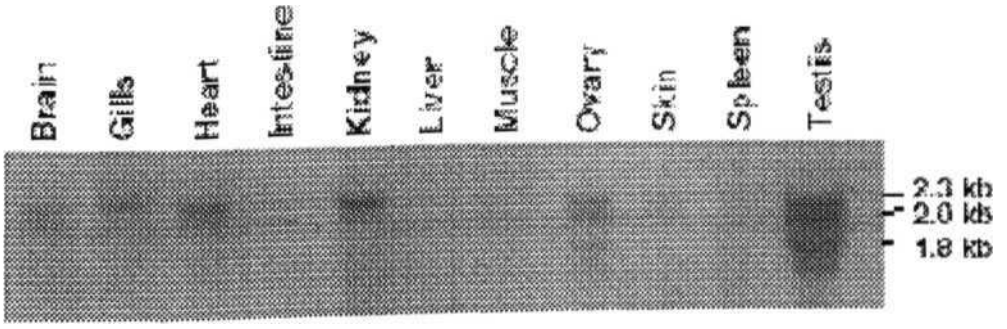


Figure. 17 : Northern blot analysis of fugu MPR 46. Fugu MPR 46 mRNA transcript detected by Northern blot analysis. 10 μg of total RNA isolated from different tissues of fugu was subjected to denaturing agarose gel electrophoresis transferred to hybond-N nylon membrane and hybridized with ^{32}P labeled fugu MPR 46 570 bp cDNA fragment. A single band of 2.3 kb mRNA transcript was seen in many tissues.

Species	Size of mature protein (kDa)	cDNA clone size (kb)	Repetitive domain sequence/ *transmembrane domain*	Conserv-ation of cystein residues	Ligand binding region sequences (in 3rd/ 9th domains for MPR 300)	Potential N-glycosylation sites	Size of RNA transcript (kb)
Bovine	300	7.497	DL(N/Q)(P/A)L	Yes	CSSCHQRMSV/CHKVYQRSTT	Conserved	~9.5
	46	*0.840*	*ILLVTLASLVAVYIIGGFLY*	*Yes*	*DNHCGREQRRA*	*Conserved*	*~2.3*
Human	300	7.473	DL(N/T/S)(P/A)L	Yes	CSSGFQRMSV/CHKVYQRSTA	Conserved	~9.4
	46	*0.837*	*ILLVTFASLVAVYVVGGFLY*	*Yes*	*DNHCGKEQRRA*	*Conserved*	*~2.3*
Mouse	300	7.446	DL(N/S)(P/V)L	Yes	CSSGFQRMSV/CHKVYQRSTT	Conserved	~10.0
	46	*0.837*	*ILLVTFASLVAVYIIGGFLY*	*Yes*	*DNHCGKEQRRA*	*Conserved*	*~2.3*
Goat	300	Partial	DL(N/Q/S)(P/A)L	Yes	CSSGFQRMSV/-	Conserved	~9.3
	46	*0.840*	*ILLVTFASLVAVYIIGGFLY*	*Yes*	*DNHCGREQRRA*	*Conserved*	*~2.3*
Chicken	300	7.410	DLSPL	Yes	CSSGFQRMTV/CHKIYERSTA	Conserved	~11.0
	46	*Partial*	*ILLITFSALVTVYIVGGFLY*	*Yes*	-	*Conserved*	*~3.3*
Fugu	300	7.488	DLS(P/R)L	Yes	CSSGFERMTI/CHKIYQRSTE	Conserved	~9.5
	46	*0.819*	*IILIIGFCLLTVYLVGGFLY*	*Yes*	*GS-CSKDASQA*	*Conserved*	*~2.3*

Table.7 : Structural similarity among MPR proteins (MPR 300 & MPR 46) in the vertebrates.

DISCUSSION

Mannose 6-phosphate receptor proteins (MPR 300 and MPR 46) have been extensively characterised from liver tissues of mammals and different cell lines (Suresh *et al* , 2004). In addition, the chicken receptors have also been well characterised (Matzner *et al,* 1996). First, biochemical evidence for the appearance of both receptors among different non-mammalian vertebrates such as the reptiles, amphibians and fish came from our studies (Siva kumar *et al,* 1997; 1999). The receptors have alos recently been identified in the invertebrate mollusc, *unio* (Siva kumar and von figura, 2002).

The comparison of the primary sequences of the MPR 300 protein among all vertebrates as discussed in the preceding chapter indicates that there exists homology in the 15 repetitive cassette structures of all vertebrate MPR 300 proteins. In addition, the luminal domain of MPR 46 exhibits 14-37% similarity to the 15 repetitive cassettes of the MPR 300 among the mammals (Lobel *et al,* 1988). This finding led to the assumption that MPR 46 is the most ancient in evolution and MPR 300 arouse by multiple gene duplication from MPR 46.

Among the different mammalian MPR 46 proteins the primary sequences of human, bovine, mouse, and rat receptor are known (reviewed in, Dahms and Hopcock, 2002). Among the non-mammalian vertebrates so far only a partial cDNA sequence of the chicken MPR 46 (cytoplasmic tail sequence) is available. Comparison of the cytoplasmic tail sequences of the chicken cell MPR 46 to the mammalian MPR 46 proteins indicated extensive structural homology in the cytoplasmic tail (Matzner *et al,* 1996). Since fish, the earliest vertebrate has been known to contain the putative MPR 46, (Siva Kumar *et al,* 1999) we undertook the present study to get the full length cDNA clone for this protein and to sequence the same to understand the structure and evolution of this receptor.

In the present study we isolated a full length cDNA clone for the fugu MPR 46 which was completely sequenced. The results obtained indicate that the initiation codon (AAGATGG) is within the published consensus sequence (Kozak *et al,* 1981; 1986). A careful analysis of the structural features of the fugu MPR 46 in comparison with other known mammalian MPR 46 proteins suggests the following common structural features. i) The appearance of the 5' UTR region, followed by the signal sequence, ii) The amino terminal region consisting of four potential glycosylation sites, iii) the highly conserved transmembrane domain, iv) conserved transport signal sequences in the cytoplasmic tail, v) the stop codon is present with the sequence TGA and vi) the GGA binding region that helps in sorting process which has been recently identified in mammals (Rosa Puertollano *et al*, 200; Yunxiang Zhu et al, 2001) is also present in the fugu 46 protein.

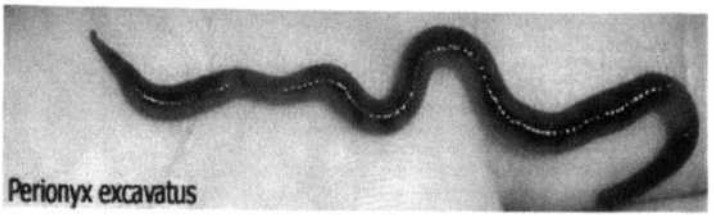
The only puzzling feature is the missing arginine residue in the ligand binding site of the receptor that is known for bovine and human receptors. Some explanation that can possibly be given is that the fish receptor has been shown by us earlier to bind to phosphomannan Sepharose gel only in the absence of sodium chloride and in the presence of three divalent metal ions (10 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$), where as the mammalian 46 receptors (bovine, human, goat, and others, bind to phosphomannan Sepharose in presence of sodium chloride and 10mM $MnCl_2$). Consistent with this, another representative receptor of the non-mammalian vertebrates (chicken cell MPR 46) also exhibits the same property (Udaya lakshmi *et al*, 2000). Since the amino terminal region sequences of the CEF MPR 46 are not available, it is difficult to make any structural comparison of this receptor to the fugu 46. From the existing data, it is logical to conclude that possibly in fugu other amino acids might play a crucial role in binding. With the sequence information available for the fugu 46 its expression in MPR 46 deficient cells and analysis of its function, and further site

directed mutagenesis would precisely define the ligand binding residues of the fugu MPR 46. To obtain further confirmatory evidence that the fish receptor is indeed present in other fish species, we also isolated a full length cDNA clone for MPR 46 from the RNA isolated from *xiphophorus xiphidium* cell lines. We earlier detected MPR 46 protein from these cells (Siva Kumar et al., 1999). The sequence of the *xiphophorus* MPR 46 also shows extensive structural homologies to other known MPR 46 and to the fugu 46 protein.

In summary this is the first report on the isolation of a full length cDNA sequence of the earliest non- mammalian vertebrate fish fugu. The study clearly demonstrates the following : First, the earliest vertebrate fish, has both the putative MPR 300 and 46 proteins, that exhibit structural similarity to other vertebrate MPR 300 and 46 proteins known. Second, it further supports our earlier studies on the identification of the putative receptor proteins in fish, suggesting that the putative receptors are present in all vertebrates thereby strengthening the basis for the evolution of the receptor proteins. Third, the transmembrane domain is highly conserved. Fourth the transport signals identified for mammalian MPR 46 proteins in the cytoplasmic tail are also highly conserved. Taken together, this study reports on the first isolation of the full length cDNA clone of the fugu MPR 46 protein. Expression of the full length sequence of fugu MPR 46 in MPR 46 deficient cells and studying its function would throw light on the functions of this protein, which is the future direction of our work.

CHAPTER IV

IDENTIFICATION OF MPR 300 LIKE PROTEINS IN ARTHROPODA AND ANNELIDAE



PENAEUS INDICUS
PHYLUM : ARTHROPODA
SUBPHYLUM: CRUSTACEA
CLASS: MALOCOSTRACA
ORDER: DECAPODA

PERIONYX EXCAVATUS
PHYLUM: ANNELIDA
CLASS : OLIGOCHAETA
ORDER: LUMBRICULIDA

INTRODUCTION

The existence of two homologous mannose 6-phosphate receptors (MPR 300 and **MPR** 46) 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. Therefore, it is important to carry out this study in order to understand the evolution of these interesting proteins.

Earlier employing an affinity matrix Sepharose-divinyl sulfone-phosphomannan developed in our laboratory, we have identified both putative mannose-6-phosphate receptor proteins from goat liver and from different non-mammalian vertebrates such as the Reptiles, Amphibians and Fish (Siva kumar et al; 1997., 1999). In the present study we have successfully isolated cDNA clones for the fish MPR proteins thus demonstrating the existence of putative receptors throughout vertebrates. Homologous receptors were also identified from the invertebrate *unio* employing the phosphomannan gel. (Udaya lakshmi et al; 1999., Siva kumar and von figura; 2002). Additionally, in a recent study we were able to detect the putative receptors and also an a- fucosidase enzyme which is possibly lysosomal in origin from the molluscs *(unio).*

The objective of the present study was to use the same affinity gel to first identify whether the **MPR** 300 protein is present among other invertebrates such as Arthropods (*Peneaus Indicus)* and Annelidae (*Perionyx Excavatus)* which are below the molluscs in the animal kingdom. We also explored the possibility of identifying the a- fucosidase enzyme (a known mammalian lysosomal enzyme) from arthropods.

MATERIALS

Instruments:

Heating block thermostat	Bangalore geneie
y- counter	ECIL, Hyderabad
Pipetman 20,200,1000 uL	Glaxo
Spectrophotometer,UV	Spectronic
Western blot transfer unit	BioRad

Chemicals:

Acetone	SQL
Acetonitrile, HPLC	Baker
30% Acrylamide/ 0.8% Bisacrylamide (NN' methylenebisacrylamide)	Qualigens
Acetic acid	SQL
Ammonium acetate	Qualigens
BCA	Sigma
BSA	Sigma
Bromophenol blue	BioRad
Calcium chloride ($CaCl_2$	Qualigens
Chloroform	SQL
Dimethylsulfoxide ultra pure (DMSO)	SQL
PPO	SQL
DTT	Serva
EDTA	Qualigens
Ethanol	SQL
Ethanolamine	Sigma
Formaldehyde (37%)	Sigma
Freund's complete/ incomplete adjuvant	Sigma
Glucose 6-phosphate	Sigma
Glycerin	Merck
Glycine	SQL
Guanidium hydrochloride	Fluka

HPLC grade water	Baker
HEPES	Sigma
Imidazole	Sigma
Iodoaceticacid (1AA)	Serva, Heidelburg
Iodogen	Pierce Co.
^{125}T	BRIT
Liquid Scintillation fluid	Roth
P-M.E	Sigma
Methanol	SQL
Pansorbin cell suspension (Heat inactivated *staphylococcus aureus* cell suspension)	Calbiochem
Paraformaldehyde	Sigma
Phenylmethylsulfonylfluoride (PMSF)	Serva, Heidelburg
Prestained high molecular weight protein markers	Sigma
Propylgallat (3,4,5-Trihydroxybenzoic acid propylester)	Serva
Saponin	Sigma
Sepharose 6B	Sigma
SDS	Sigma
Silver nitrate	Qualigens
TEMED	Sigma
TCA	Merck
TFA	Fluka
Tris-(hydroxymethyl)-aminoethane(Tris)	Qualigens
Triton X-100	Sigma
Tween 20	Sigma

Animal tissues are obtained from local market and transferred to lab on ice.

Methods:

Hydrolysis of *O*-phosphomannan Y-2448:

Hydrolysis of *O*-phosphomannan Y-2448 was done according to the method of Bretthauer *et al* (1973). 2.5 g of O-phosphomannan (from yeast *Hansenula holstii*, a generous gift from Dr. M.E.Slodki, USD A, IL, USA) was suspended in 500 mL of distilled water in a screw cap bottle and allowed for swelling overnight at room temperature. To the suspension 500 mg of KC1 was added and the pH was adjusted to 2.4 with acetic acid. Then the suspension was hydrolyzed in a boiling water bath at 100°C for 1 hour. Then it 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 Ba $(OH)_2$. Equal volume of 95% ethanol was added to the above suspension and incubated overnight at 4 °C. This was centrifuged at 10,000 RPM for 30 min. The phosphomannan core (PM) that was pelleted was dissolved in water and acidified with acetic acid and dialyzed in water and lyophilized.

Preparation of Affinity matrix (PM gel):

10 mL of Sepharose 6B gel was washed extensively with distilled water over a sintered glass funnel and was suspended in 10 mL of 0.5M sodium carbonate buffer pH 11.0. 1.0 mL of divinyl sulfone was added to the gel and the suspension rotated end over end for 70 min at room temperature. The gel was washed extensively with water and was suspended in sodium carbonate buffer pH 10.0 containing 250 mg of phosphomannan core (PM). Coupling was allowed to proceed overnight at 4°C on a rotator. The gel was washed extensively with water and suspended in carbonate buffer pH 8.5 containing 0.2 mL of β-mercaptoethanol .The gel was rotated end over end at room temperature for 3 hours. After this the gel was washed with water and suspended in column buffer and stored at 4° C until further use.

Acetone powder preparation:

This was prepared according to the method described by Distler and Jourdian (1987). The whole animal tissue of Arthropod (without exoskeleton) and Annelidae was homogenized in a mixer with 1.6 volume of 0.5 mM $CaCl_2$ and 1 mM $NaHCO_3$. The pH was adjusted to 5.0 by drop wise addition of 4 N acetic acid. The suspension was centrifuged at 9000 RPM for 15 minutes. The pellet was homogenized with 6 volumes of chilled acetone and filtered rapidly through whatmann 3 MM filter paper in a buchner funnel. The cake obtained was washed with chilled diethyl ether. The cake was dried on ice over an aluminium foil. The dry powder obtained was stored at -80 °C until use.

Extraction of Membrane Proteins :

All operations were carried out at 4 °C with the following buffers

Buffer 1 - 50 mM imidazole-HCl pH 7.0, 150 mM NaCl, 2 mM PMSF.

Buffer 2 - 50 mM sodium acetate buffer pH 4.6, 150 mM NaCl, 0.5 mM $CaCl_2$.

Buffer 3 - 50 mM imidazole-HCl pH 6.5, 5 mM sodium β-glycerophosphate.

Buffer 4 - Buffer 3 containing 0.05% Triton X100 and 10 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$.

Acetone powders prepared above for each species of Arthropod and Annelidae were homogenized with 6 volumes of buffer 1 in a mixer and stirred overnight at 4 °C. The suspension was centrifuged at 9000 rpm for 20 minutes and the supernatant discarded. The pellet obtained was resuspended in 6 volumes of buffer 2 and homogenized well in a mixer and centrifuged at 9000 rpm for 20 minutes and the supernatant discarded. The pellet was suspended in 6 volumes of buffer 3 and homogenized in a mixer. The suspension was stirred overnight at 4 °C after addition of DOC and Triton X 100 to a final concentration of 0.1% and 1%, respectively. The suspension was centrifuged at

4000 rpm for 15 minutes and the pellet discarded. To the clear supernatant obtained $MgCl_2$, $MnCl_2$, $CaCl_2$ were added to a final concentration of 10 mM each and the suspension stirred for 1 hour at 4 °C. The suspension was centrifuged at 9000 rpm for 45 minutes. The clear supernatant obtained containing membrane proteins served as a source for MPRs.

Affinity chromatography on PM gel :

The membrane proteins obtained from fresh whole animal tissue or acetone powder was passed through Sepharose-DVS-PM affinity matrix which was pre-equilibrated with column buffer (50 mM imidazole-HCl pH 6.5, 5 mM sodium β-glycerophosphate, 0.05% Triton X 100 and 10 mM each of $MgCl_2$, $MnCl_2$, $CaCl_2$) at 4°C. The gel was washed extensively with the column buffer and the bound proteins were eluted with two bed volumes of 5 mM glucose-6-phosphate to ensure any non specific elution of protein, followed by two bed volumes of 5 mM mannose-6-phosphate in column buffer. The eluates were concentrated using amicon concentrator.

Protein estimation :

Protein estimation was done using BCA reagent with BSA as standard following manufacturer's instructions.

BCA reagent : 10 mL of BCA and 0.5ml of 4% copper sulfate.

The volume of the protein sample was made upto 500 µL with distilled water and mixed with 1.0 mL of BCA reagent and incubated for 30 minutes at 37°C. After incubation the absorbance was measured at 562 nm.

SDS-Poly Acrylamide Gel Electrophoresis:

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

Tank buffer:	3g Tris, 14g Glycine and 1g SDS dissolved in 1 litre of double distilled water
2x Sample buffer : (reducing)	0.5M Tris-HCl pH 6.8 2.5 mL, 10% SDS 4.0 mL, 100% glycerol 2.0 mL, β-mercaptoethanol 1.0 mL, bromophenol blue 0.05% and made up the volume to 10 mL
Resolving gel buffer:	1.5 M Tris-HCl pH 8.8
Stacking gel buffer:	0.5M Tris-HCl pH 6.8
Acrylamide:	30% (w/v) Acrylamide, 0.8% (w/v) N N' methylenebisacrylamide

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

Resolving gel and stacking gels are made as shown in the **Tables.8** and 9, respectively. APS and TEMED must be added at the end to resolving gel and poured into the sealed glass plates (mini gel). After polymerization the gel was rinsed with water. Stacking gel solution was poured, a comb with required number of wells was 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 briefly and the supernatant loaded into the wells. These were overlaid with tank buffer and placed in the electrophoretic chamber. Samples were subjected to electrophoresis for 2-3 h at 50 100 V at room temperature.

% of PAA gel	7.5%	10%	12.5%
H_2O (mL)	3.650	3.025	2.400
Resolving gel buffer (mL)	1.875	1.875	1.875
Acrylamide (mL)	1.875	2.500	3.125
10% SDS (µL)	75	75	75
APS (µL)	40	40	40
TEMED (µL)	10	10	10

Table.8 : Reagents used for the preparation of resolving gel with different **polyacrylamide** concentration.

Reagents	Volume
H_2O (mL)	1.875
Acrylamide (mL)	0.375
Stacking gel buffer (mL)	0.3125
10% SDS (µL)	25
APS (µL)	40
TEMED (µL)	8

Table.9 : Reagents used for the stacking **gel.**

Silver **Staining :**

Blum *et* ***al.***, (1987)

Reagents: Methanol, Ethanol, $AgNO_3$, Hypo, Na_2CO_3, Formaldehyde **and** Acetic acid.

All the following steps were carried out at room temperature by placing the gel on a rocking platform.

1. Fixative: 50% methanol, 12% glacial acetic acid, 50 µL of formaldehyde (HCHO) were taken in a flask and the volume made up to 50 mL with double distilled water. The gel was soaked for 45min to overnight. (Fixative can be stored in a brown bottle and can be reused 4-5 times).

2. The gel was transferred to 50% ethanol and incubated for 1 hour.

3. The gel was rinsed 3 times with double distilled water.

4. 10 mg Hypo was dissolved in 50 mL of double distilled water. The gel was soaked exactly for 1 min. in the above solution with constant shaking.

5. The gel was rinsed 3 times with double distilled water.

6. 100 mg $AgNO_3$ was dissolved in 50 mL of double distilled water. To this 18 µL of formaldehyde was added. The gel was soaked in the above solution for 20 min. with constant shaking.

7. The gel was rinsed 3 times with double distilled water

8. Developer: 3 g of Na_2CO_3 was dissolved in 50 mL of distilled water. To this 26 µL of formaldehyde was added. The gel was soaked in this solution to visualize the protein bands.

9. The gel was rinsed with double distilled water.

10. The developing reaction was stopped by the addition of 12% glacial acetic acid.

The gel was washed twice with double distilled water and dried in a gel dryer.

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).

This α-MSC1 antiboby was kindly provided by Prof. Kurt von Figura , Goettingen, Germany.

MPR 300 Rabbit a-goat MPR 300 antibodies (Udaya lakshmi and Sivakumar.,1996)

Rabbit frog MPR 300 antibody and

Rabbit *Unio* MPR 300 antibodies (Siva kumar and von figura; 2002)

Antibodies to the purified receptors :

Antibodies to the affinity purified MPR 300 protein from goat, frog and unio were available in the laboratory. To obtain MPR 300 specific IgG from these antisera, a goat-receptor affinity gel was prepared.

Preparation of goat MPR 300-receptor **affigel :**

Affigel-10 was processed following the manufacturer's instructions. 1.0 mL of Affigel-10 was packed in a sintered syringe and washed consecutively with cold isopropanol, water and 0.1 M HEPES buffer pH 7.5. Purified goat MPR 300 protein in 1.0 mL of 0.1 M HEPES buffer pH 7.5 was added to the washed Affigel. The column was closed and the gel suspension rotated for 24 hours at 4 °C. At the end of the incubation period, the unbound fraction was collected, acidified and the protein measured at A_{280} Unreacted sites in the gel were blocked using 200 uL of 0.1 M ethanolamine hydrochloride pH 8.0 for 1 hour at 4 °C and the gel was washed thoroughly with water followed by PBS.

Purification of MPR 300 specific IgG on **receptor affigel :**

1.0 mL of goat-affigel prepared above was equilibrated with 10 mL of 10 mM Tris-HCl pH 7.4 containing 150 mM NaCl (column buffer). In separate experiments 10 mL of the

goat, frog and *unio* antisera were applied on the gel. The gel was washed extensively with column buffer and eluted with 100 mM glycine-HCl pH 2.65 buffer and 1.0 mL fractions were collected. The eluted fractions were neutralized with 2 M Tris, pooled, concentrated and used in experiments.

Iodination of Proteins :

Fraker and Speck (1978)

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

Iodo-gen (1,3,4,6-tetrachloro 3a, 6a-diphenyl glycoluril) solution : 0.8 mg of iodogen (M.W 432.06) was directly weighed into an eppendorf tube and dissolved in 577 µL of dichloromethane. 200 µL of it was transferred to another tube and diluted 1-3 times to get final 1 mM concentration.

Iodogen tubes : 50-80 µL of iodogen solution was uniformly coated under nitrogen atmosphere at the bottom of a conical glass centrifuge tube (Iodogen rapidly initiates iodination, the thin film of iodogen on the reaction tubes minimizes the direct exposure of the protein to the oxidant).

Column buffer: 1mg/mL potassium iodide, 0.05% BSA, 0.05% Triton X 100 in 10 mM PBS.

Mannose-6-phosphate eluates from PM gel were collected and concentrated in an amicon concentrator (10 kDa cut off membrane). The concentrated protein was acetone precipitated with chilled (-20 °C) acetone at a final 80% concentration and incubated overnight at -20 °C. The sample was centrifuged at 14,000 RPM for 10 minutes, supernatant removed and the protein pellet was air dried by leaving the tubes open on ice. The pellet was suspended in 60 µL of borate buffer and sonicated on ice to disrupt

any aggregates. The sample was centrifuged briefly and the clear supernatant transferred to a fresh conical glass tube numbered as tube 1. To this 1uL (100 μci) of ^{125}I was added under the hood and the tube was closed with para film and incubated on ice for 5 minutes. The contents were transferred to tube 2 (iodogen tube) and incubated with rotation on ice for 8 minutes. The reaction was stopped by transferring the contents to tube 3 containing 10 uL of 20 mM β-mercaptoethanol. 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 500uL.

The iodinated protein fraction was separated from free iodine by passing the sample on a 5.0 mL gel filtration G-25 column pre-equilibrated with 20 mL of column buffer. The sample was applied on the column and 10 fractions each of 0.5 mL were collected in eppendorf tubes. 2 uL from each fraction was taken in fresh tubes and counted for 1 minute in a y-ray counter. Peak tubes corresponding to the protein were pooled, and used in subsequent experiments.

Repurification on PM gel :

1.0 mL of PM gel was packed in a 5.0 mL sintered column and equilibrated with column buffer (50 mM imidazole-HCl pH 6.5, 5 mM sodium β-glycerophosphate, 0.05% Triton X 100, and 10 mM each of $MgCl_2$, $MnCl_2$, $CaCl_2$). A fixed amount of iodinated receptor corresponding to 100,000 CPM was taken in an eppendorf tube, the volume was made upto 500 uL with column buffer and applied on to the column. Unbound fraction was collected, followed by 10 wash fractions each of 500 uL. The fractions were counted in γ-ray counter, until the counts were equal to background counts. Elution was done first with 5 mM glucose 6-phosphate (5 fractions of 500 uL each were collected) followed by 5 mM mannose 6-phosphate (5 fractions of 500 uL each were collected). 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 immunoprecipitation and SDS-PAGE.

TCA Precipitation of Iodinated Protein:

The iodinated protein sample was taken in an eppendorf tube, the volume was made up to 100 μL with column buffer and 72% TCA was added to a final 10% concentration. This was incubated on ice for 30 min. The sample was centrifuged at 14,000 rpm for 10 min and supernatant was transferred to another tube. Both pellet and supernatant were counted in a γ-ray counter. Pellet was then suspended in 20-30 μL of 1 M Tris and equal volume of 2x sample buffer (+/- SH buffer) was added and cooked at 95°C for 5 min. The samples were centrifuged briefly and subjected to SDS-PAGE analysis. The gel was put in the fixative (50% methanol, 10% acetic acid in water) for 30-60 min, washed thrice with water, dried and exposed overnight to Roentgen film at -70°C.

Immunoprecipitation of the Iodinated MPR Protein:

A fixed number of counts (100,000 cpm) of the iodinated receptor were taken into two eppendorf tubes labeled as PI (pre immune) and IM (immune). To this 2 μL of pre immune serum or immune serum (for MPR 300) or 10 μg of affinity-purified MSC1 antibody (for MPR 46) was added and the volume was made up to 400 μL with PBS-Tween buffer (10 mM 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 1.0 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.

***Dictyostelium Discoideum* lysosomal enzyme secretions Affigel :**

Preparation of Affigel :

Affigel-10 was processed following the manufacturer's instructions. 1.0 mL of Affigel-10 was packed in a sintered syringe and washed consecutively with cold isopropanol, water and 0.1M HEPES buffer pH 7.5. *Dictyostelium Discoideum* lysosomal enzyme secretions were dissolved in 1.0 mL of 0.1 M HEPES buffer pH 7.5 and added to the washed Affigel. The column was closed and the gel suspension rotated for 24 hours at 4 °C. At the end of the incubation period, the unbound fraction was acidified and the protein concentration measured at A_{280}. Unreacted sites in the gel were blocked using 200 µL of 0.1 M ethanolamine hydrochloride pH 8.0 for 1 hour at 4 °C and the gel washed thoroughly with water followed by PBS.

Radio iodinated samples from both arthropod and annelidae species were extensively dialysed against column buffer (50 mM imidazole-HCl buffer pH 7.0, 5 mM sodium P-glycero phosphate, 0.05% Triton X 100, 5 mM EDTA and applied on affigel coupled with *Dictyostelium Discoideum* lysosomal enzyme secretions. Washed with column buffer and eluted with 5 mM glucose-6-phosphate followed by 5 mM mannose-6-phosphate in column buffer.

Purification of α-fucosidase :

Enzyme assay **for** a-fucosidase :

To check the activity of a-fucosidase the following assay was done:

Reagents : 50 mM sodium acetate buffer pH 5.0, 0.2 M sodium carbonate buffer pH 9.2

Substrate: 5 mM para nitrophenyl a-D-fucopyranoside dissolved in

50 mM sodium acetate buffer pH 5.0.

Volume of the sample is made upto 400 µL with 50 mM sodium acetate buffer pH 5.0. To this 100 µL of substrate was added and incubated for 15 minutes at 37^0C. The reaction was stopped by adding 500 µL of 0.2 M sodium carbonate buffer pH 9.2. The absorbance of the sample was then measured at A405. The activity of the enzyme is calculated using the formula

$$\text{Activity} = \frac{\text{absorbance at 405nm}}{\text{(Units/mL) Time of incubation x 18.5 x volume of enzyme}}$$

Isolation and purification of a-fucosidase enzyme :

100 grams of fresh arthropod tissue was taken and homogenized in a mixer with 4 volumes of 25 mM Tris-HCl buffer pH 8.0 and stirred for 3 hours to overnight at 4 °C. The suspension was centrifuged at 12,000 rpm for 15 minutes and the pellet was discarded. The clear supernatant was passed through DE-52 Cellulose gel column (50 mL) pre-equilibrated with column buffer (25 mM Tris-HCl pH 8.0). The column was washed with column buffer until no more protein was eluted from the gel. Bound proteins were eluted with column buffer containing 0.2 M NaCl. The enzyme containing fractions were pooled. Volume of the pooled fractions was measured and ammonium

sulfate was added to a final concentration of 1 M. This was then passed through Phenyl-Sepharose gel (20 mL) pre-equilibrated with column buffer (25 mM Tris-HCl pH 8.0, 1 M ammonium sulfate). The column was washed extensively with column buffer and the proteins were eluted with column buffer without ammonium sulfate. The enzyme containing fractions were pooled. Volume of the pooled fractions was measured and ammonium sulfate was added to 80% saturation, so as to concentrate the proteins. The suspension was stirred for 2 hours and centrifuged at 12000 rpm for 15 minutes. The supernatant was discarded and the pellet was dissolved in 10 mM sodium phosphate buffer pH 5.5 and dialysed against the same buffer.

The dialyzed sample was passed through gel filtration column G-200 (1 cm x 90 cm) pre-equilibrated with 10 mM sodium phosphate buffer pH 5.5. 3.0 mL fractions were collected and the enzyme containing fractions were pooled.

The pooled fractions were passed through L-Fucose gel (SIGMA f-3902) pre-equilibrated with column buffer (10 mM sodium phosphate buffer pH 5.5). The column was then washed until the A_{280} was 0.05. Bound protein was eluted with 10 mM α-L-fucose in column buffer. The active fractions were pooled and dialyzed against column buffer to remove the ligands.

Western blot analysis:

This was carried out according to Towbin *et al.*, (1979). A 10 % SDS-PAGE gel was run with the purified α-fucosidase and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% NFM in TBS buffer pH 7.4 for 1 hour. The membrane was incubated with antiserum of *unio* α-fucosidase as primary antibody, overnight in TBS buffer pH 7.4 containing 1% NFM. The membrane was washed with TBS Tween followed by TBS and blocked for 5 minutes with 5% NFM in TBS buffer.

The membrane was later incubated with secondary antibody, goat-anti rabbit IgG conjugated with alkaline phosphatase for 1 hour in TBS buffer containing 1% NFM. The membrane was washed with TBS Tween followed by TBS and then with distilled water. The blot was developed with BCIP/NBT substrate until the bands were visualized and then the blot was washed with water to stop the reaction. The membrane was air dried and photographed.

RESULTS

Affinity purification of MPR like protein from Arthropoda and Annelidae :

The membrane protein extracts from the arthropod and annelidae whole animal tissues were subjected to affinity chromatography on PM gel in the presence of divalent metal ions (Ca^{+2}, Mn^{+2}, Mg^{+2}). After washing the column extensively, the protein was eluted with 5 mM mannose-6-phosphate. SDS-PAGE analysis of the eluates showed a faint band similar to MPR 300 in size, which was detected in both the samples.

Later, the PM eluates from both the species were subjected to amicon concentration (10 kDa cutoff membrane) and the proteins were concentrated. The concentrated proteins were acetone precipitated and radio iodinated with ^{125}I as described under methods. The radioiodinated samples were subjected to SDS-PAGE under reducing conditions for Arthropod species and Annelidae species.

PM repurification :

The radio iodinated arthropod and annelidae proteins were reapplied on PM gel as described under methods. The column was washed and the bound proteins were eluted with 5 mM G6P followed with 5 mM M6P. The results shows that the proteins from arthropod and annelida could be eluted specifically with M6P and not with G6P **(Fig.18).**

***Dictyostelium discoiedium* lysosomal enzymes coupled affigel :**

The radio iodinated arthropod and annelidae proteins were also applied on *Dictyostelium discoiedium* lysosomal enzyme secretions coupled affigel as described in the methods. The gel was washed and the bound proteins were eluted with 5 mM G6P followed with

5 mM M6P. All the radioactivity applied was detectable in the unbound fraction suggesting that the proteins did not bind to the affigel under these conditions **(Fig. 19).**

Immuno precipitation with **MPR** 300 antibodies :

The radio iodinated arthropod and annelidae proteins were immunoprecipitated with MPR 300 antibodies of goat, frog and *unio* (which were prepared as described under methods). The immuno precipitates were analyzed on SDS-PAGE and detected by fluorography (Fig.20). The results showed no bands corresponding to MPR 300 were detected with the above antibodies in arthropod and annelida species.

Immuno precipitation with a MSC_1 antibody :

The radio iodinated arthropod and annelidae proteins were immuno precipitated with a MSC_1 antibody as described in methods. The immuno precipitates were analyzed on SDS-PAGE and detected by fluorography **(Fig.21)**. The results showed no bands corresponding to MPR 46 were detected with the a MSC_1 antibody in arthropod and annelida species.

Purification of **α-fucosidase from arthropod** species :

α-fucosidase was purified by passing the whole tissue extract of arthropod through different affinity columns. First, the extract was passed through DE-52 column and the eluates are collected **(Fig.22).** The active fractions for a-fucosidase of DE-52 eluates were pooled, applied on phenyl-sepharose column and eluates were collected (Fig.23). The active fractions of phenyl-sepharose eluates were pooled, concentrated with 80% ammonium sulfate precipitation and dialyzed. The dialyzed sample was passed through a G-200 gel filtration column and the active fractions were pooled. These fractions were

applied on L-Fucose gel column and the bound α-fucosidase was eluted **(Fig.24)** and aliquot of the sample was analysed on a SDS-PAGE and silver stained. A band corresponding to 56 kDa was observed which is similar to *unio* α-fucosidase in molecular weight.

Western blot analysis of arthropod a-Fucosidase :

Eluates from phenyl-Sepharose gel and L-fucose column were subjected to SDS-PAGE and the proteins were transferred to nitrocellulose membrane. The membrane was incubated with antiserum *of unio* α-fucosidase as primary antibody. The membrane was later incubated with secondary antibody, goat -anti rabbit lgG conjugated with alkaline phosphatase and developed with BCIP/NBT reagent. A band corresponding to 56 kDa was observed in the blot (Fig.25) corresponding to arthropod a-fucosidase.

Figure.18 : 10 % SDS-PAGE of radiolabeled Arthropod and Annelidae proteins repurified on PM gel.

10% SDS-PAGE under reducing conditions. Repurification of Arthropod (A) and Annelidae (B) radio-iodinated MPR 300 like proteins on PM gel.

Lane 1 Unbound
Lane 2 Wash
Lane 3 Glucose 6-phosphate eluate
Lane 4 Mannose 6-phosphate eluate

Figure. 19 : 7.5 % SDS-PAGE analysis of radiolabeled Arthropoda and Annelidae proteins binding to *Dictyostelium discoideum* lysosomal enzyme secretions gel.

Binding of radio-iodinated MPR 300 like proteins from Arthropod (A) and Annelidae (B) on *Dictyostelium discoideum* lysosomal enzyme secretions gel.

Lane 1 Unbound
Lane 2 Wash
Lane 3 Glucose 6-phosphate eluate
Lane 4 Mannose 6-phosphate eluate

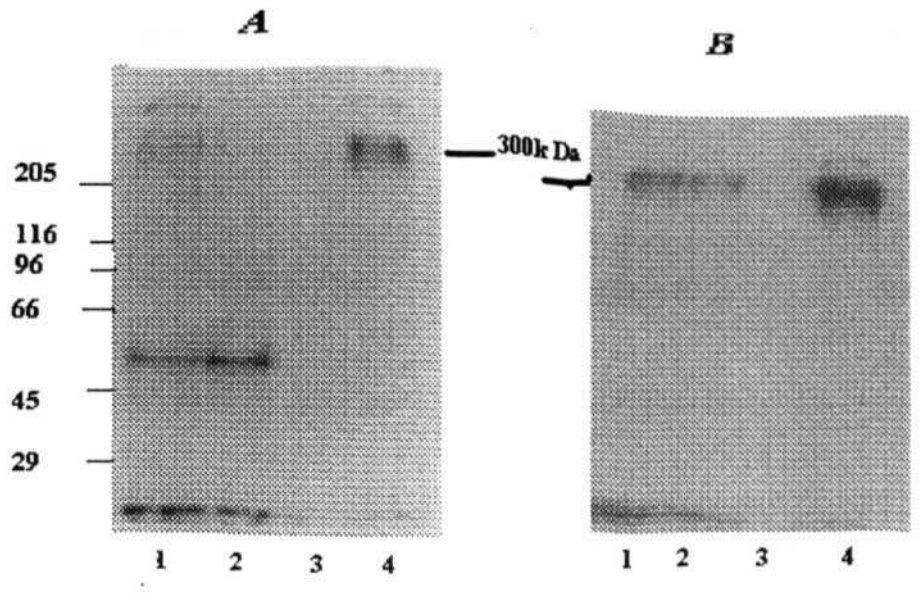


Figure. 18

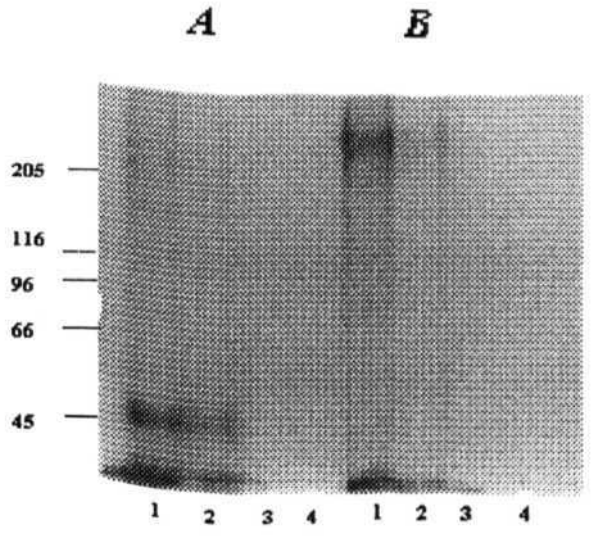


Figure. 19

Figure.20 : Immuno precipitation of the iodinated MPR 300 protein from goat, arthropod and annelidae analysed by 7.5% SDS-PAGE.
Immunoprecipitation of the iodinated MPR 300 protein from goat, arthropod and annelidae using goat, frog and unio MPR 300 antibodies.

Lane 1 Preimmune serum
Lanes 2-4 Goat iodinated MPR 300 reacted with affinity purified anti-goat, anti-frog and anti-*unio* MPR 300 antibodies
Lanes 5-7 Arthropod iodinated protein reacted with same antibodies
Lanes 8-10 Annelidae iodinated protein reacted with same antibodies

Figure.21 : Immuno precipitation of the iodinated MPR 46 protein from goat, arthropod and annelidae analysed by **10% SDS-PAGE**
Immunoprecipitation of the iodinated MPR 46 protein from goat, arthropod and annelidae using α-MSC1 antibody.
Lane 1 Pre immune serum
Lane 2 Iodinated goat MPR 46 protein immunoprecipitated with α-MSC1 antibody
Lane 3 Iodinated arthropod protein immunoprecipitated with α-MSC1 antibody
Lane 4 Iodinated annelidae protein immunoprecipitated with a-MSCl antibody

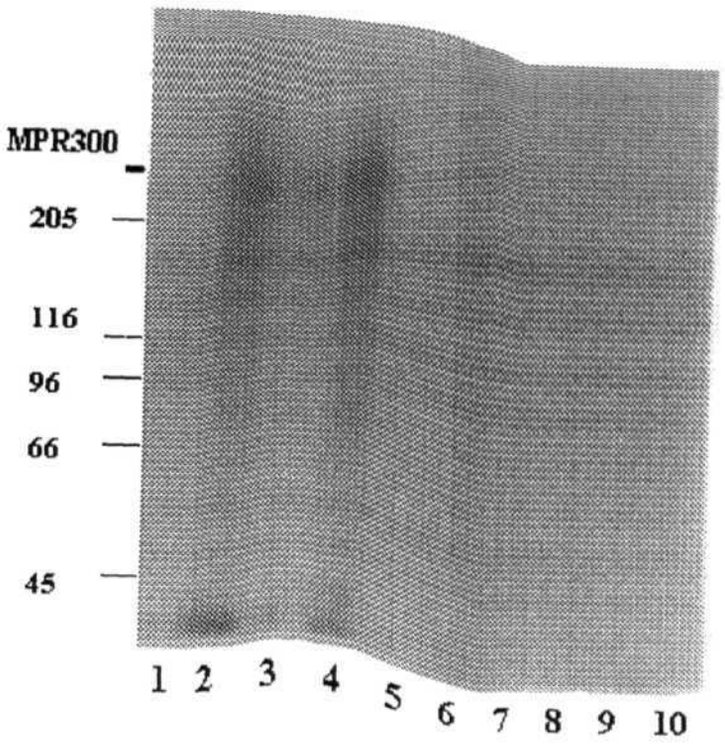


Figure.20

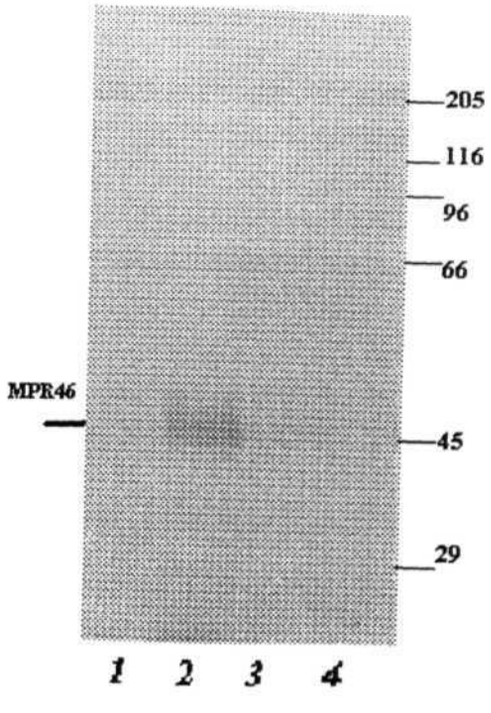


Figure.21

Figure.24 : Elution profile of the a-fucosidase from arthropod on L-fucose gel. The bound enzyme was eluted with 10 mM phosphate buffer pH 5.5 containing 10mM α-L-Fucose.

Figure.25 : Western blot analysis of the purified a-fucosidase from arthropod.

10% SDS-PAGE of the phenyl Sepharose eluate (lane 1) and the fticose gel eluate (lane 2) was carried out and the proteins transferred to nitrocellulose membrane and probed with *unio* α-fucosidase antibody.

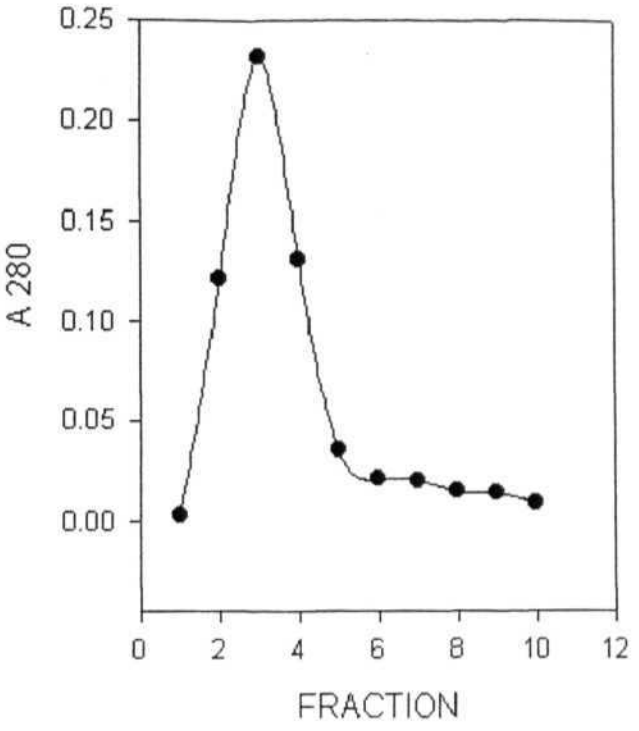


Figure.24

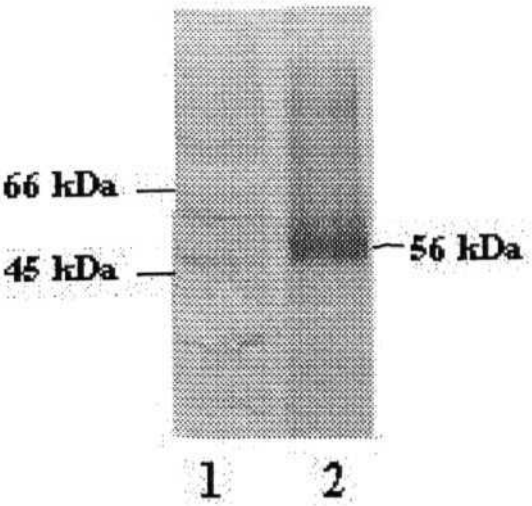


Figure.25

DISCUSSION

MPR's have been affinity purified on phosphomannan affinity chromatography. Among the two receptors MPR 300 binds to the affinity matrix independent of cations and also binds to *Dictyostelium Discoideum* lysosomal enzyme secretions gel (that are known to contain methylated mannose-6-phosphate residues) (Stein et al; 1987).

Recently employing an affinity matrix Sepharose-divinyl sulfone-phosphomannan developed in our laboratory, we have isolated both putative mannose-6-phosphate receptor proteins among the different non-mammalian vertebrates such as the reptiles, amphibians and fish (Siva kumar et al; 1999). The same gel was also found to be useful in purifying the MPR 300 protein from the invertebrate *unio* (Udaya lakshmi et al; 1999). Among the invertebrates, both Arthropod and Annelidae which are below the molluscs, contain mannose-6- phosphate receptor like protein that shows specific binding to phosphomannan-Sepharose gel and can be eluted specifically with mannose-6-phosphate. Eluted proteins on radioiodination can be rebound on PM gels and can be specifically eluted with mannose 6-phosphate. The proteins exhibit an apparent molecular mass of 300 kDa, similar to the mammalian MPR 300 protein.

The proteins from the Arthropoda and Annelidae do not bind to *Dictyostelium discoideum* lysosomal enzyme secretions gel, a property that is exhibited by the mammalian MPR 300 and also by the non-mammalian MPR 300 (Frog receptor).

The proteins from Arthropod and Annelidae do not show cross-reactivity with the mammalian, non-mammalian, as well as, invertebrate MPR 300 affinity purified antibodies. However, the antibodies raised for goat MPR 300 protein was found to cross-react with the mammalian, non-mammalian vertebrate and mollusc MPR 300 proteins. Since the Arthropod and Annelidae proteins do not cross-react with the goat antisera and also do not bind to the *Dictyostelium discoideum* lysosomal enzyme

secretions gel, the protein identified by us in both the species is therefore designated as MPR 300 like protein.

Further proteins related to MPR 46 could not be detected either in Arthropod or Annelidae when the PM eluates were immunoprecipitated with a MSC1 antibody (an antibody to the cytoplasmic tail of the human MPR 46 protein), that recognizes the MPR 46 in mammals, non-mammalian vertebrates and molluscs.

To our knowledge among the invertebrates a-fucosidase enzyme has been purified and characterized from the mollusc, *unio* (Focerelli et al; 1998). An antibody was raised for the purified enzyme in our laboratory and it was suggested that the enzyme is possibly lysosomal (Siva kumar et al; 2004).

In the present study since we identified MPR 300 like protein in the Arthropod and Annelidae we wanted to analyze whether a-fucosidase is present in the Arthropods. The experimental results indicate that the putative enzyme is present in Arthropods and it also cross-reacts with the mollusc enzyme antibody, suggesting that the invertebrate enzyme is antigenically similar. However, it remains to be established whether the enzyme in Arthropods is also lysosomal. Several lines of evidence suggest that the enzyme purified from Arthropod species is related to the mollusc enzyme. First, it efficiently binds on hydrophobic affinity gel (phenyl Sepharose) and fucose gel. Second, in SDS-PAGE it exhibits a molecular mass of 56 kDa. Thirdly it cross-reacts with the mollusc enzyme antibody.

Phylum	Species	M-6-P dependent binding to PM gel		Immunoreactivity with antibodies against mammalian	
		MPR 300	MPR 46	MPR 300	MPR 46
Vertebrates					
Mammalian	**Human,bovine, mouse,goat**	+	+	+	+
Aves	**Chicken**	+	+	+	+
Reptiles	**Garden lizard**	+	+	+	+
Amphibia	**Frog**	+	**ND**	+	+
Pisces	**Fish**	+	+	+	+
Invertebrates					
Molluscs	**Unio, Bg cells**	+	+	+	+
Arthropoda	**Prawn**	**A**	**NI**	-	-
Annelida	**Blue worm**	**A**	**NI**	-	-

Table. 10 : Current knowledge on the appearance of MPR proteins in evolution.
A -MPR like proteins that bind to PM gel and eluted with M6P.
NI- Not identified , ND - Not determined.

SUMMARY

•A full length cDNA clone of Fugu MPR 300 gene was isolated and sequenced which shows homology with known MPR 300 proteins.

•Northern blot of Fugu MPR 300 shows a 9.5 kb band corresponding to MPR 300 of other known species.

•A full length cDNA clone of Fugu MPR 46 gene was isolated and sequenced which shows homology with known MPR 46 proteins.

•Northern blot of Fugu MPR 46 shows a 2.3 kb band corresponding to MPR 46 of other known species.

•A full length cDNA clone of *Xiphophorus* MPR 46 gene was also isolated and sequenced which shows homology with known MPR 46 species.

•From Arthropoda and Annelidae, mannose 6-phosphate receptor like protein was isolated that is capable of binding to phosphomannan gels and can be specifically eluted with M6P. However, these proteins do not bind on *Dictyostelium discoideum* lysosomal enzyme secretions gel.

•The proteins from Arthropod and Annelidae do not show cross-reactivity with the mammalian, non-mammalian, as well as, invertebrate MPR 300 affinity purified antibodies. No proteins similar to the mammalian and non-mammalian MPR 46 were detectable in the animal species tested.

•A 56 kDa a-fucosidase enzyme (possibly lysosomal) was isolated from Arthropoda and it cross-reacted with an antibody to the enzyme from another invertebrate species, ***unio***.

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PUBLICATIONS

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Two separate manuscripts describing the molecular cloning of the fish receptors are under preparation.