Functional characterization of non-mammalian cation-independent mannose 6 -phosphate/insulin like growth factor-II receptor

Thesis submitted for the Degree of

Doctor of Philosophy

By

Yadavalli Siva Rama Krishna

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046
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Enrollment No: 03LBPH09 May 2008

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CERTIFICATE

This is to certify that this thesis entitled "**Functional characterization of non-mammalian cation-independent Mannose 6-phosphate/Insulin like growth factor-II receptor***"* submitted to the University of Hyderabad by **Mr. Y.SIVARAMAKRISHNA** 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.

Prof. N. Siva Kumar
Supervisor

Head
Department of Biochemistry

Dean
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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 **Prof. N. Siva Kumar**. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

Y.SIVARAMAKRISHNA

Date:

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Yadavalli SivaRamaKrishna

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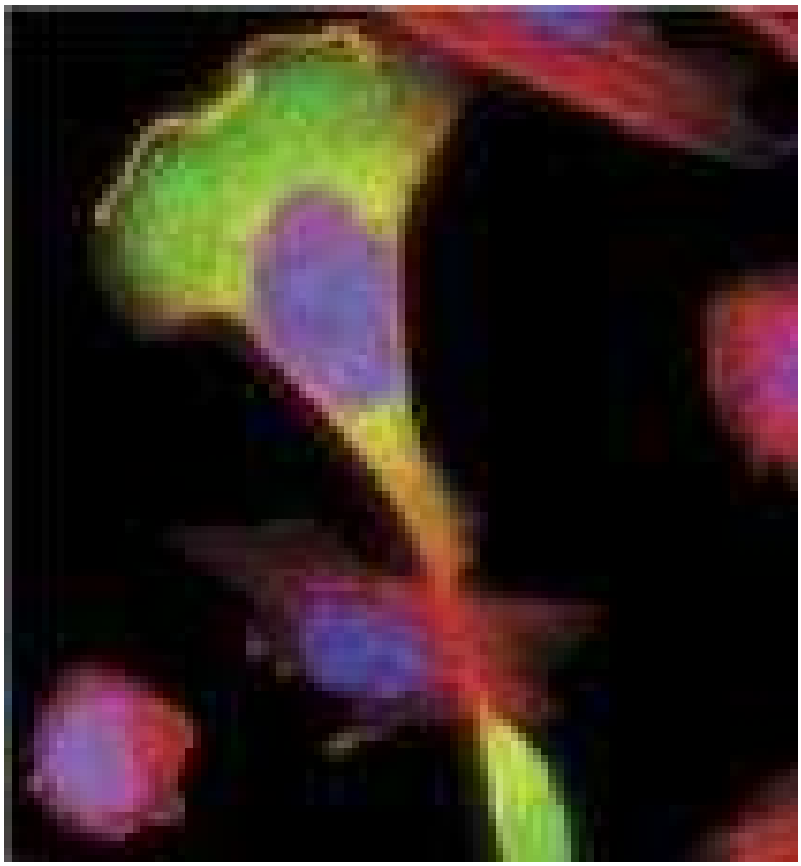

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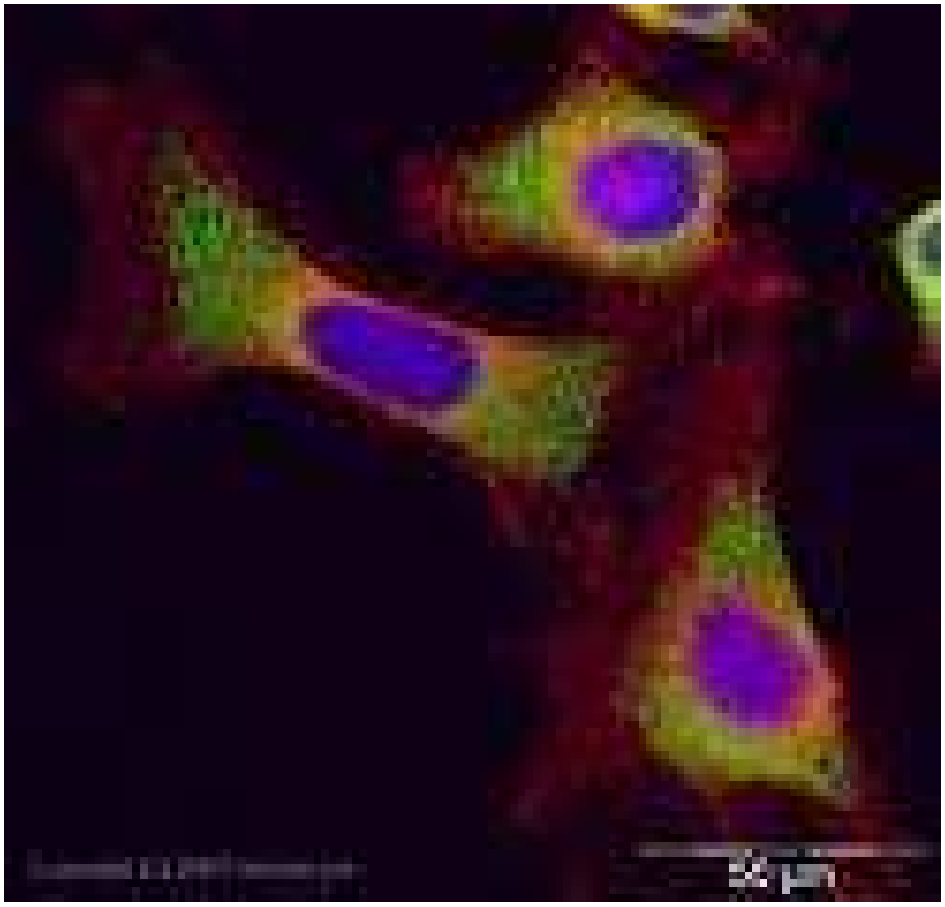

MPR 300 MEDIATES ENDOCYTOSIS OF THYROGLOBULIN ON CEF CELLS

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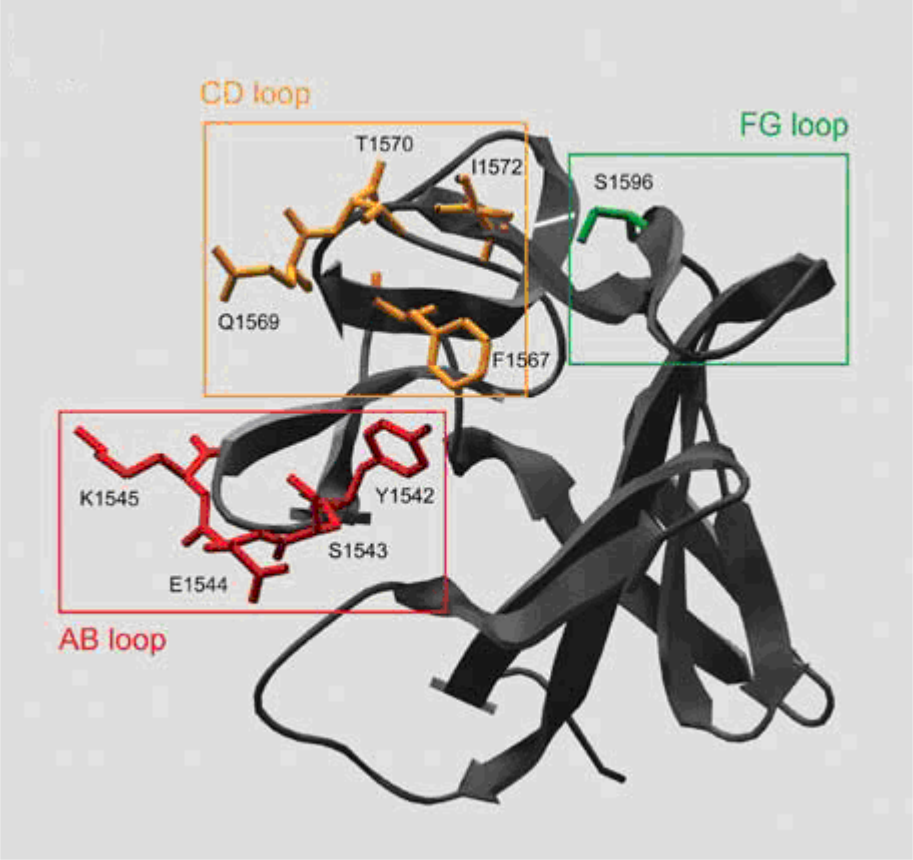

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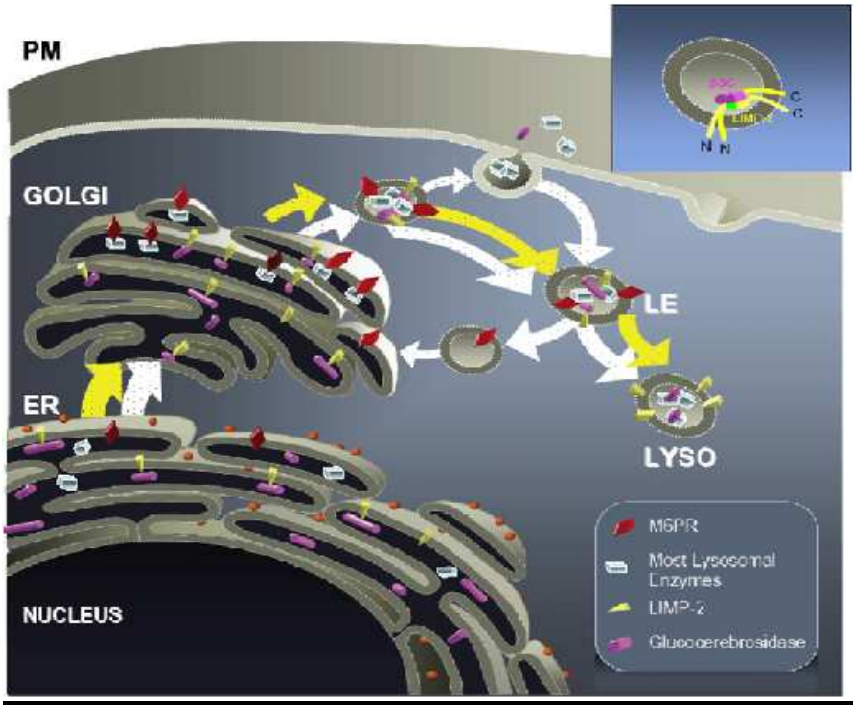

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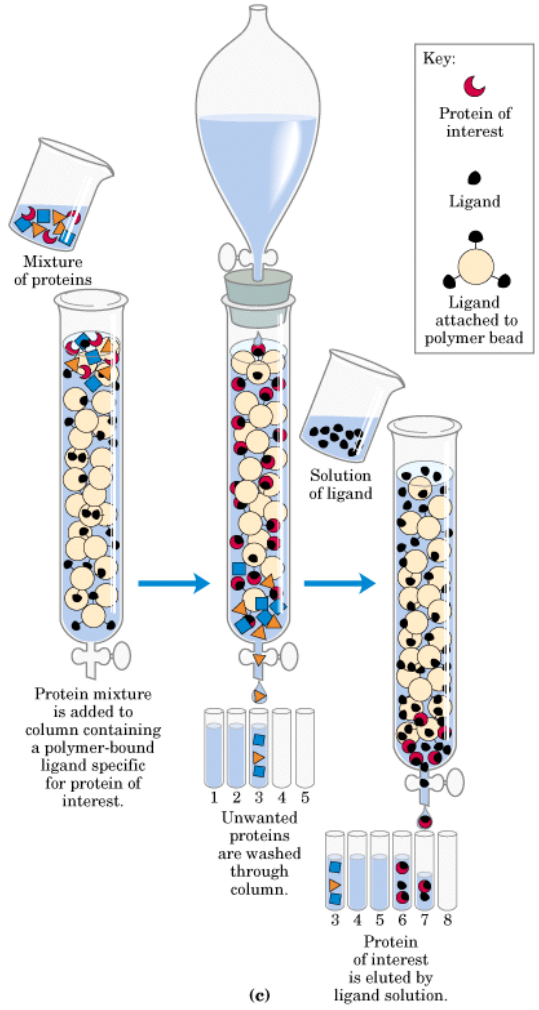

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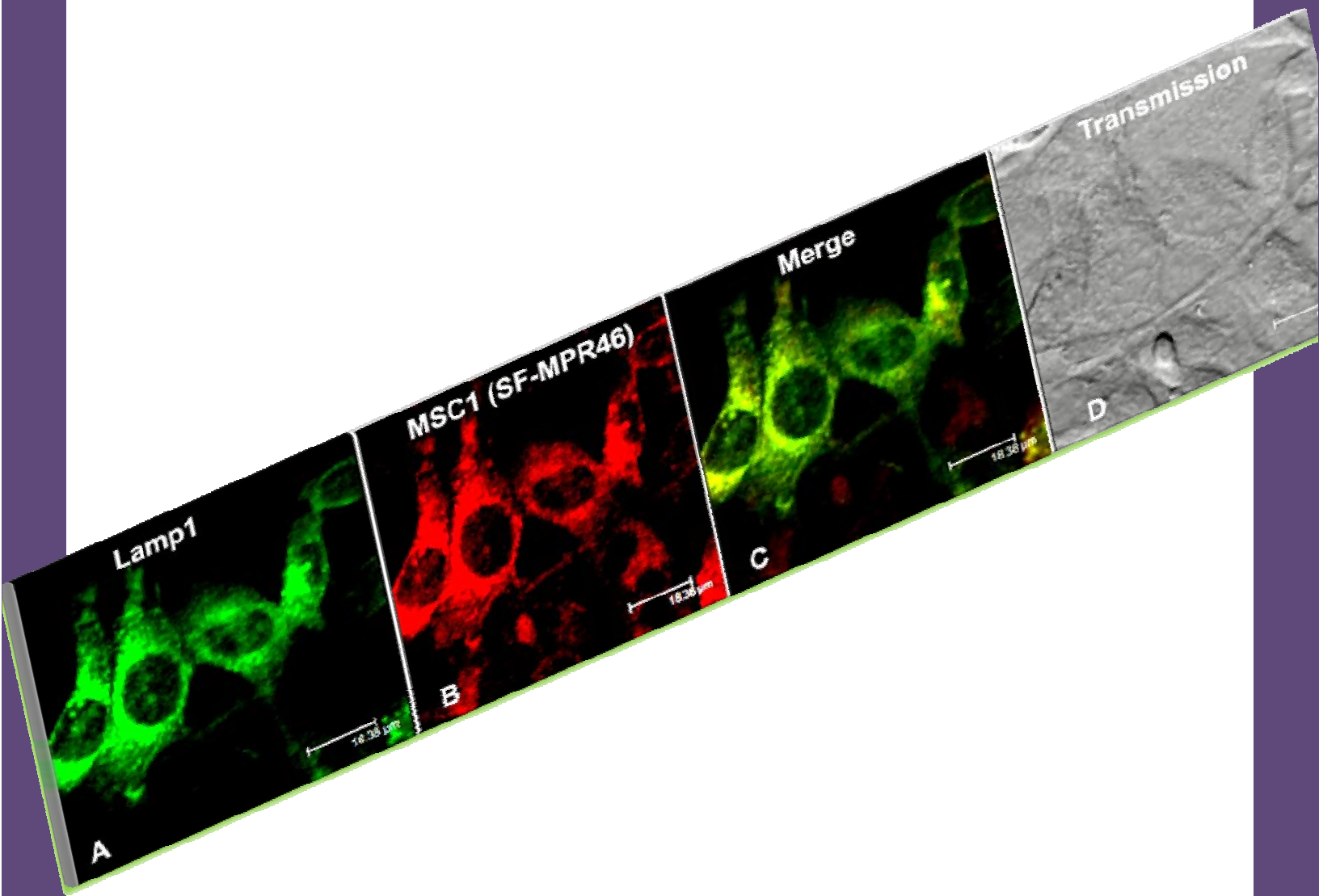

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Part-II

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Abbreviations

APS	Ammoniumperoxodisulfate
BCA	Bicinchoninic acid
bp	base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CEF	Chicken Embriyonic Fibroblast
cpm	Counts per minute
CTP	Cytidine triphosphate
dATP	Deoxyadinosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
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))
HIGF-II	Human insulin like growth factor-II
kb	Kilo basepair
kDa	Kilo Dalton
LB	Luria Bertani
M6P/IGF-IIR	Mannose 6-phosphate/insulin like growth factor II receptor
Man 6-P	Mannose 6-phosphate

min	Minute
MOPS	Morpholino propane sulfonic acid
MPR	Mannose 6-phosphate receptor
nm	Nanometer
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
PVDF	Polyvinyldifluoride
R_f	Relative front
RNAi	RNA interference
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
Taq	*Thermophilus aquaticus*
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylendiamine
TFA	Trifluroacetic acid
TGN	*trans*-Golgi Network
Tg	Thyroglobulin
T_M	Melting temperature
Tris	Tris-(Hydroxymethyl) aminoethane
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UV	Ultraviolet
β-M.E	β -mercaptoethanol
mCi	micro Curie

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Lysosomes were first described by De Duve and his collaborators, as cellular organelles filled with acid hydrolases (De Duve, 1983) a discovery that won him the Nobel Prize. Lysosomes are membrane bound organelle localized in the cytoplasm of most cells and function in intracellular digestion. They digest materials taken into the cell from outside (a process known as endocytosis) as well as other materials that originate in the cell's own cytoplasm (autophagy). The materials to be digested are ultimately incorporated into the same membrane-bound compartments as the lysosomal enzymes. Selective degradative products can pass out of the lysosome by crossing the membrane, but the enzymes cannot. This sequestration, which protects the cell, persists because the admixture of the enzymes and the materials to digest takes place through fusion of membrane-bound compartments.

1. Lysosomes and lysosomal enzymes

1.1 Structure and function of lysosomes

Lysosomes are intracytoplasmic organelles defined by an acidic milieu (pH around 4.5) and surrounded by a single membrane that is present in all cell types in mammals except red blood cells. They are connected with the endocytic network and are implicated in the digestion of macromolecules. For this purpose, lysosomes contain numerous enzymes, probably more than fifty, allowing the degradation of proteins, nucleic acids, polysaccharides, lipids and their conjugates. All these substrates originate both from endocytosis and autophagy (Fig.1.1). Autophagy and endocytosis are connected lysosomal pathways because auto phagosomes receive inputs from endocytic vesicles during their maturation (Dunn, 1994; Cuervo, 2004; Majeski et al., 2004). Lysosomal acidic hydrolases comprise a variety of proteases, nucleases, glycosidases, sulfatases and lipases. Lysosomal (degradative) function also involves some other proteins such as integral membrane proteins, saposins,

and yet uncharacterized proteins. Additionally, lysosomes have been now considered as organelles that can play an important role in numerous biological processes in eukaryotes such as antigen presentation by MHC II molecules, bone resorption, tumour progression and programmed cell death (Dell'Angelica et al., 2000). In some cell types of the immune system and melanocytes, specialized lysosomes secrete cytotoxic molecules or melanin (Blott et al., 2002; Stinchcombe et al., 2004).

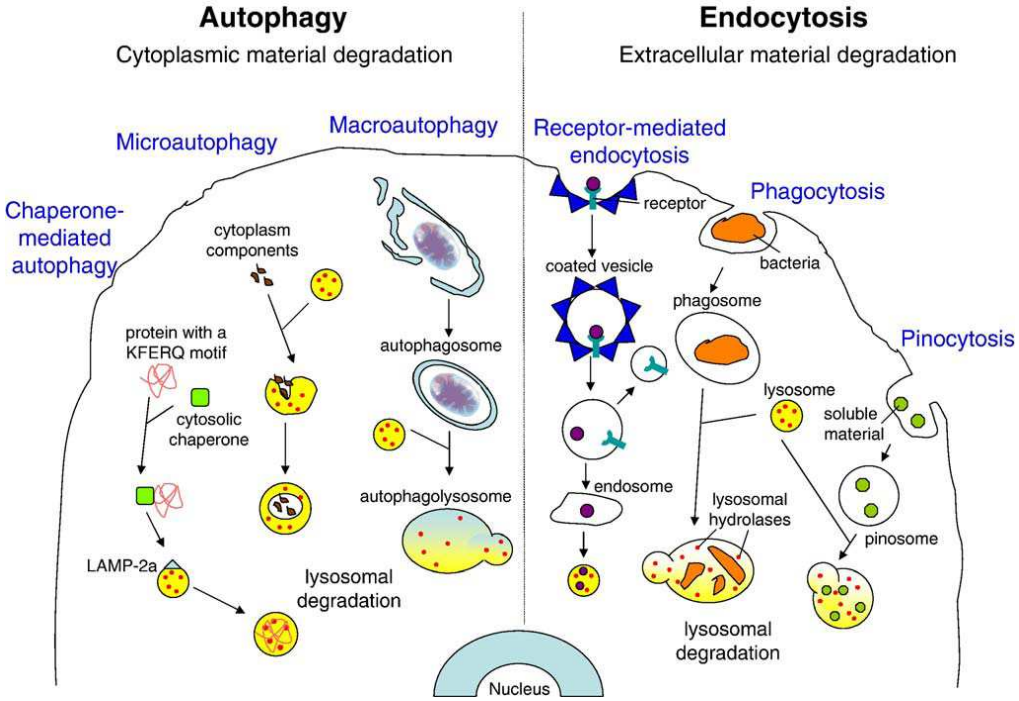


Fig. 1.1 **Different pathways leading to degradation by lysosomes**. Endocytosis provides lysosomes with extracellular material for digestion. It comprises three distinct processes: (i) phagocytosis, that results in the digestion of particulate material such as bacteria and occurs only in certain specialized cells like neutrophils and macrophages, (ii) pinocytosis, that allows for internalization of soluble material, and (iii) receptor-mediated endocytosis, in which the recognition of a molecule by its cognate membrane receptor is required to lead to its engulfment. The autophagic process is implicated in the degradation of cytoplasmic constituents and is generally activated in response to stress conditions. Three types of autophagy can be distinguished: (i) macro autophagy, which involves the formation of a double membrane vesicle that fuses with the lysosomal compartment, (ii) micro autophagy, which consists in the sequestration of cytosolic components directly at the surface of the degradative organelle, and (iii) chaperone-mediated autophagy that targets to the lysosomal membrane substrate proteins having a peptide motif related to KFERQ which is recognized by a cytosolic molecular chaperone. Binding to the lysosome-associated membrane protein type 2a (LAMP-2a) is then followed by translocation of the substrate protein to the lysosomal lumen.

Interest in lysosomes and lysosomal enzymes was stimulated by the existence of some 30 inherited lysosomal storage disorders in man. The enzyme defects involved in most of these disorders were identified in the 1970s (Neufeld et al., 1975). Presently, these mutations are being characterized at the level of DNA and RNA. Targeting of lysosomal enzymes is part of the more general question: how do eukaryotic cells transport proteins synthesized in the rough endoplasmic reticulum to diverse destinations? Hickman and Neufeld discovered, in 1972, that the multiple deficiency of lysosomal enzymes in I-cell disease results from a deficiency in a recognition marker that is common to lysosomal enzymes and required for targeting the enzymes to lysosomes. This observation provided the basis for many subsequent studies that eventually led to the identification of the recognition marker (a phospho-transferase enzyme that is responsible for addition of phosphate residues to mannose on the enzymes) and its receptor. The first receptor to be identified was a 215-kd protein, which recognizes mannose 6-phosphate residues in lysosomal enzymes, has been identified as an essential component of a system which in many cells allows the specific transport of lysosomal enzymes to lysosomes. It was originally identified as a cell surface receptor binding exogenous lysosomal enzymes and mediating their transfer to lysosomes along the pathway of receptor-mediated endocytosis. We now know that this receptor functions also in transport of endogenous lysosomal enzymes and that its presence in organelles that constitute elements of the secretory pathway is important for that function. The combined application of biochemical and cytological methods has significantly contributed to the present knowledge of lysosomal enzyme transport. Further, the current application of recombinant DNA methods to the study of lysosomal enzymes and their receptors is expected to provide answers to many unresolved questions.

1.2 Role of the Man 6-P/IGF-II receptor in the transport of lysosomal enzymes

The mannose 6-phosphate receptors (215 kDa protein, and the second receptor identified later, 46 kDa protein to be discussed below) are responsible for directing the transport of proteins possessing a mannose 6-phosphate recognition marker to the lysosomes. Newly synthesized lysosomal enzymes bind to the mannose 6-phosphate receptors in the *trans*-Golgi Network. These complexes are then localized into coated pits of the Golgi apparatus and coated vesicles are formed. These vesicles then uncoat and enter a compartment, called the pre-lysosomal compartment (PLC) or compartment of uncoupling of receptor and ligand (CURL), where the vesicle acidifies and lysosomal enzymes are released. A further sorting occurs in this compartment such that the lysosomal enzymes are directed to the lysosomes and the receptors return to the Golgi apparatus (von Figura and Hasilik, 1986; Dahms et al., 1989). Although the transport of lysosomal enzymes is understood in mammals, the precise role of the two mannose 6-phosphate receptors in this process has not been clearly defined as yet in non-mammalian species. However, only the Man 6-P/IGF-II receptor is capable of binding to mannose 6-phosphate containing proteins at cell surface to direct them to lysosomes (Sly et al., 1982; Kyle et al., 1988). Further experiments identified the recognition marker to be sensitive to oxidation (Hickman et al., 1974), mannosidase treatment (Hieber et al., 1976), deglycosylation and alkaline phosphatase treatment (Ulrich et al., 1978). Uptake of lysosomal enzymes was inhibited by a number of agents, but more strongly by fructose 1-phosphate, mannose 6-phosphate, and phosphomannans (Sando et al., 1977; Kaplan et al., 1977a; Kaplan et al., 1977b; Ulrich et al., 1978). The recognition marker was eventually identified as mannose 6-phosphate as this sugar inhibited the uptake of some enzymes by cells. These include β-galactosidase

(Distler et al., 1979) or N-Acetyl-glucosaminidase (von Figura et al., 1979) or β-glucuronidase (Natowicz et al., 1979) that are known to contain mannose 6-phosphate.

The synthesis of the mannose 6-phosphate recognition marker on lysosomal enzymes is a two step process (Lazzarno et al., 1988). Briefly, N-acetylglucosaminyl l-phosphate is transferred from UDP-N-acetyl glucosamine to the C6 hydroxyl of a mannose residue by UDP-N-acetyl-glucosamine: lysosomal enzyme N –acetyl glucosaminylphosphotransferase. The N-acetyl-glucosamine is then removed by N-acetyl glucosamine 1- phosphoexoester α-N-acetylglucosaminidase to create the mannose 6-phosphate recognition marker (von Figura and Hasilik, 1986). The transferase prefers to phosphorylate at 6th position in mannose and shows selectivity for lysosomal enzymes in their native conformation (Reitman et al., 1981). While no consensus sequence for the transferase has been identified in lysosomal enzymes, the available evidence suggests that several small areas which are separated in the primary structure but near each other in the mature, folded protein may be the recognition signal for this enzyme (Faust et al., 1989). It is this process that is defective in I-cell fibroblasts due to a defective transferase (Hasilik and Neufeld, 1980).

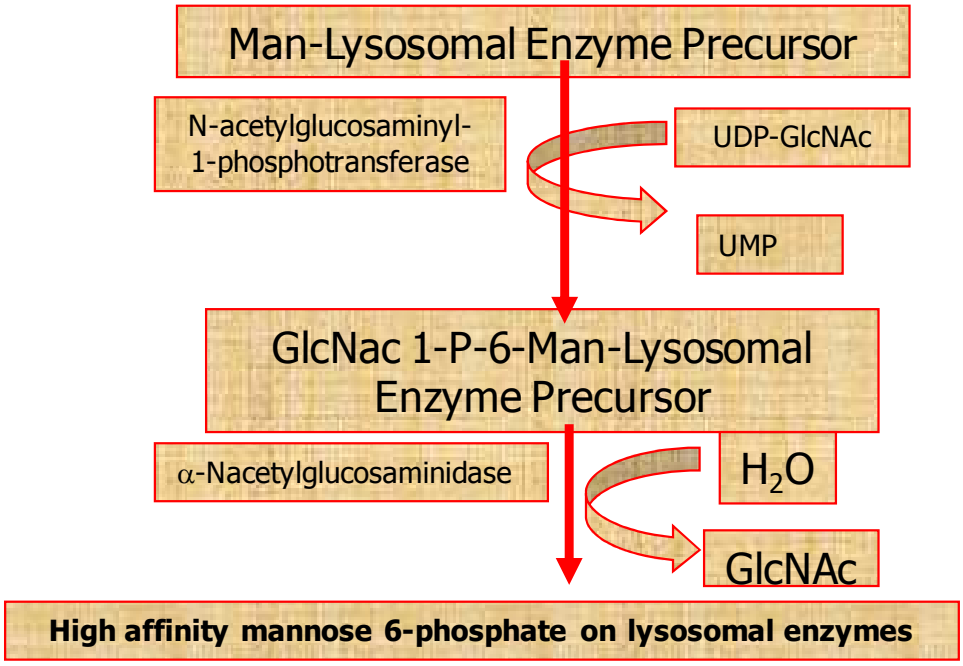


Fig.1.2 Two step biosynthetic process for the generation of terminal mannose 6-phosphate recognition marker in lysosomal enzymes.

1.3. Primary Structure of MPRs

Both mannose 6-phosphate receptors have been cloned and sequenced at the cDNA level from various species (Table 1.1). Both receptors are integral membrane proteins with three distinct domains, the extracytoplsmic domain, the transmembrane domain and the cytoplasmic domain (Fig. 1.3; Hille-Rehfeld, 1995; Pohlmann, 1996).

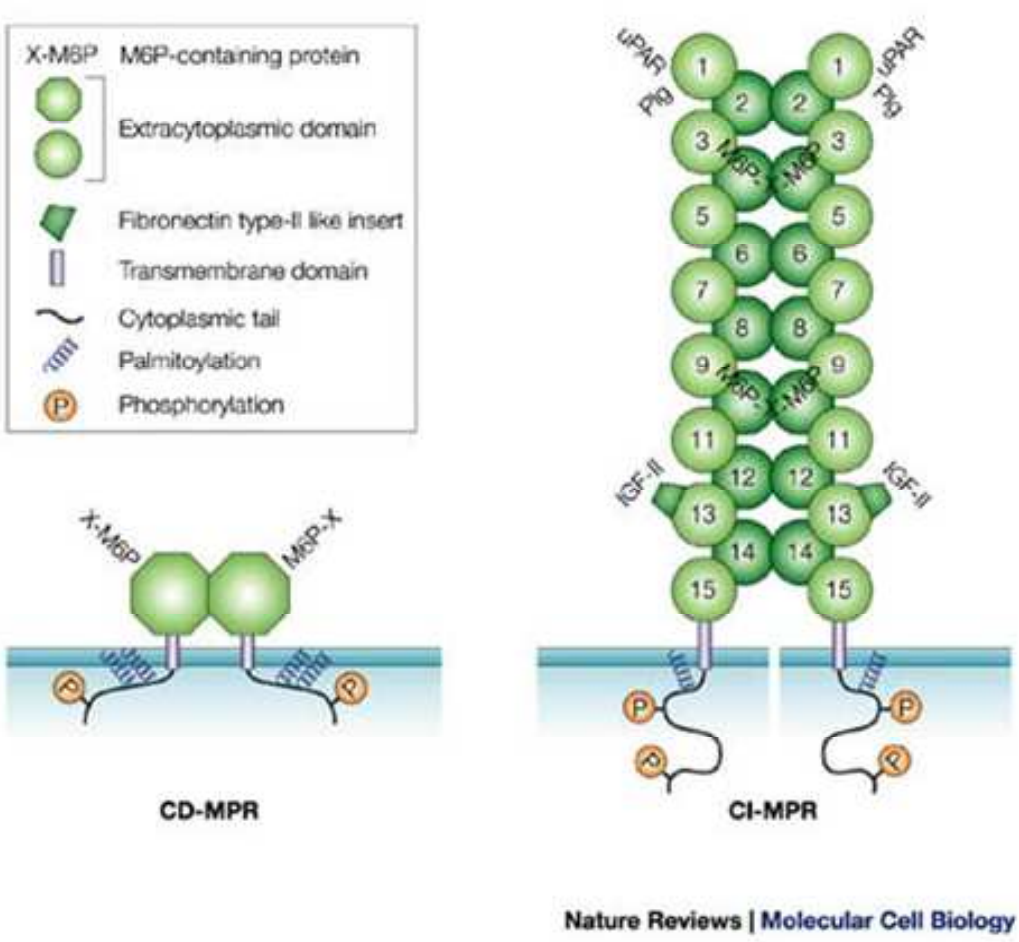


Figure 1.3: Schematic Representation of the Primary Structure of Mannose 6-phosphate Receptors. The repetitive units in the extracytoplasmic domain of MPR 300 are numbered from 1-15. The *in vivo* functional existence of monomeric and dimeric forms of MPR 300 and MPR 46 respectively are represented (according to Dahms et al., 1989).

1.4 M6P/IGFII receptor

The M6P/IGF-II receptor is a type I transmembrane glycoprotein consisting of four structural domains: a 40–44 residue amino-terminal signal sequence, an extracytoplasmic domain of 2264–2269 residues, a single 23 residue transmembrane region, and a carboxy-terminal cytoplasmic tail of 163–164 residues. The extracytoplasmic domain consists of 15 repeating segments of approximately 147 amino acids each, sharing 14–38% sequence identities (Fig. 1.3) (Dahms and Hancock, 2002). The 13th repeat contains an insertion of a 43 amino acid region

with homology to the fibronectin collagen binding domain that may influence ligand binding. The extracytoplasmic domain contains 19 potential N-glycosylation sites, of which at least two are utilized in forming the mature receptor of 275 – 300 kDa (Dahms and Hancock, 2002). Cysteine residues located in the extracellular repeating segments of the receptor form intermolecular disulfide bonds required for proper receptor folding. Other posttranslational modifications, such as phosphorylation and palmitoylation have also been reported for the receptor (Hille-Rehfeld, 1995; Pohlmann, 1996). The cytoplasmic domain of the receptor contains four regions that are known to be potential substrates for various protein kinases including protein kinase C (PKC), cAMP-dependent protein kinase, and casein kinase I and II (Korner et al., 1995; Dahms and Hancock, 2002). The available data indicate that receptor dimerization can occur both *in vitro* and *in vivo*. Furthermore, the observation that binding of β-glucuronidase increases the internalization rate of iodinated IGF-II and iodinated β-glucuronidase, suggests a mechanism in which receptor dimerization, resulting from the binding of a multivalent ligand, alters the kinetics of M6P/IGFII receptor internalization at the cell surface (Byrd et al., 2000; Hassan et al., 2003; Dahms and Hancock, 2002). A truncated form of the receptor lacking primarily the intracellular and transmembrane domains has been identified in bovine serum and in the serum, urine and amniotic fluid of rats and humans (Dahms and Hancock, 2002) formation of the soluble M6P/IGF-II receptor, which retains its ligand-binding properties, is suggested to be a mechanism for receptor turnover (Clairmont and Czech, 1989, Canfield and Kornfeld, 1989). However, several lines of experimental evidence suggest that the soluble receptor functions as a carrier protein to sequester excess free IGF-II molecules in the circulation (Zaina et al., 1998; Dahms and Hancock, 2002)

1.5 Genomic organization and expression

The genomic structure of the M6P/IGF-II receptor has been analysed for the mouse and the human. Whereas the mouse M6P/IGF-II receptor gene is located on chromosome 17 (Hille-Rehfeld, 1995; Laureys et al., 1988), the human gene has been mapped to chromosome 6 (Killian et al., 1999; Laureys et al., 1998). The total size of the human receptor gene is estimated to be 136 kb and comprises of 48 exons (Killian et al., 1999). Unlike other multi-domain receptors, such as the human low-density lipoprotein receptor, the exon boundaries of the M6P/IGF-II receptor do not correspond to its functional or structural domains: exons 1–46 encode for the extracellular region of the receptor with each of its 15 domains encoded by portions of three to five separate exons (Hille-Rehfeld, 1995). A 54-bp enhancer, comprised of two E-box motifs, and putative binding sites for the transcription factor Sp1 and NGF-1A have been identified within the 266-bp promoter region (Liu et al., 1995). The mouse M6P/IGF-II receptor gene is maternally imprinted in peripheral tissues (Barlow et al., 1991; Laureys et al., 1988). The M6P/IGF-II receptor is ubiquitously expressed in cells and tissues, but a number of studies have demonstrated that the expression level of this receptor is both tissue-specific and developmentally regulated (Dahms and Hancock, 2002). DNA methylation of the promoter region in the parental allele of the M6P/IGF-II receptor is believed to account for its suppression in peripheral tissues.

1.6 MPR 46

Hoflack and Kornfeld in 1985 first discovered a cation dependent mannose 6-phosphate receptor with an apparent molecular mass of 46 kDa. The cDNA for the MPR 46 has been cloned and sequenced from several species such as human, bovine, mouse and goat (Suresh et al., 2004 and the references therein), Fugu and

xiphoporous fish (Raju thesis, 2004) Zebra fish (Suresh et al., 2006) and partially sequenced from chicken (Matzner et al., 1996). Only recently in our laboratory the full length chicken MPR 46 gene was cloned (Praveen Kumar and Siva Kumar, unpublished information). The mRNA of the human MPR 46 codes for a protein of 277 amino acids consisting of four structural domains viz., a N-terminal signal sequence of 20 or 26 amino acids, extracytoplasmic domain of 164-170 amino acids exposed at the plasma membrane or oriented to vesicle lumen, a single transmembrane domain of 20 amino acids followed by 67 amino acids of cytoplasmic domain. The short extracytoplasmic domain was shown to exhibit 14-37 % homology to individual repeats of MPR 300 (Dahms et al., 1987, Lobel et al., 1988). In contrast to this homology, there are no sequence similarities among the signal sequences, transmembrane regions and the cytoplasmic domains of the two receptors. MPR 46 contains 5 potential N-glycosylation sites, four of which are used (Wendland et al., 1991). The protein shows an absolute requirement of divalent metal ions for ligand binding and has also been classed as the cation dependent receptor (CD receptor). The carbohydrate portion contributes to 40% of the apparent molecular mass of the MPR 46. The position of cysteine residues which are most likely involved in disulfide bond formation is well conserved within the extracytoplasmic domain of MPR 46 (Lobel et al., 1988). MPR 46 is a highly conserved protein with 93-95% overall homology from mouse to man and with completely identical amino acid sequence within the cytoplasmic domain of these species. The cytoplasmic domain contains a single casein kinase-II phosphorylation site. The gene for the human MPR 46 has been localized to chromosome 12 (Pohlmann et al., 1987). The gene spans about 12 kb and consists of 7 exons (Klier et al., 1991).

Table 1.1: Protein Structure and Genomic Organization of Mannose 6-phosphate Receptors (Hille Rehfeld, 1995)

	MPR 46	MPR 300
Primary Structure		
cDNA Cloning	1. Human (Pohlmann *et al*., 1987) 2. Bovine (Dahms *et al.,* 1987) 3. Murine (Koester *et al*., 1991., Ludwig *et al*., 1992; Ma *et al*., 1992) 4. Goat (Suresh et.al.,2004) 5. Chicken (Matzner *et al*., 1996) 6. Fugu and *Xephoporous* (Raju thesis., 2004) 7. Zebra fish (Suresh et al.,2006) 8. Starfish (present study,2008)	1. Human (Morgan *et al*., 1987; Oshima *et al*., 1988) 2. Bovine (Lobel *et al*., 1987; Lobel *et al*., 1988) 3. Rat (Mac Donald *et al*., 1988) 4. Mouse (Szebenyi and Rotwein., 1994) 5. Goat (Suresh et.al.,2004) 6. Chicken (Zhou *et al*., 1995) 7. Fugu (Raju thesis., 2004) 8. Zebra fish (Catherine et al.,2006)
Deduced amino acid sequence		
(Human MPR 46) signal sequence extracytoplasmic domain transmembrane domain cytoplasmic domain		
Internal repeats within extracytoplasmic domain	277 amino acids in total 20-26 164-170 20 67 1	2491 amino acids in total 40 2264 23 164 15 (~ 147 amino acids each)
M_r of polypeptide Apparent M_r		Fibronectin binding site in collagen showing –homology in repeat 13 M6P-binding site in repeat 3 and 9 IGF-II binding site in repeat 11
Post-translational Modifications		
	30 kDa	270 kDa
N-glycosylation sites Potential sites Glycosylated sites	43-46 kDa	275-300 kDa
Disulfide bonds		
Oligomerization	5	19
Phosphorylation	2 high mannose 2 complex	
Palmitoylation	3 pairs	3-4 pairs per repeat
	dimers, tetramers	monomer, oligomer ?
Genomic Structure	ser 56 (CK II)	ser 82 (CK II) ser 157 (CK II)
Location on chromosome	not known	yes
Gene length	12 (human) 6 (murine)	6 (human) 17 (murine)
Exon structure	12 kb 7 exons in total	130 kb 48 exons in total

1.7 Functions of the M6P/IGF-II receptor

1.7a. M6P/IGF-II receptor and intracellular sorting of lysosomal enzymes

M6P-dependent transport of soluble lysosomal enzymes is a crucial step in the biogenesis of lysosomes. Newly synthesized lysosomal enzymes are carried to the lysosomes by vesicular transport from the endoplasmic reticulum, through the Golgi complex and endosomes. Initial transport steps are shared with proteins of the secretory pathway and apparently do not require specific signals. At the *trans* face of the Golgi complex, soluble lysosomal enzymes bind M6P receptors by their M6P-recognition signal and are subsequently transported via clathrin-coated vesicles to late endosomes (also termed prelysosomes) wherein enzyme release is triggered by the acidic interior (Hille-Rehfeld, 1995; Kornfeld, 1992). The enzymes are then transported to the lysosomes by capillary movement and M6P receptors are either targeted to the cell surface or carried back to the Golgi complex (Kiess et al., 1994; Dahms and Hancock, 2002). The segregation and transport of lysosomal enzymes is believed to be mediated by both M6P receptors as they target overlapping but distinct populations of lysosomal proteins (Kornfeld, 1992; Hille-Rehfeld, 1995; Pohlmann et al., 1995). However, several lines of evidence suggest that the M6P/IGF-II receptor is more efficient than the MPR 46 protein (also termed as the cation dependent mannose 6-phosphate receptor) in effectuating the intracellular sorting of newly synthesized lysosomal enzymes (Kornfeld, 1992; Ludwig et al.,1994., Hille-Rehfeld, 1995), It is of interest to note that, in keeping with their role in the intracellular sorting of lysosomal enzymes, the majority of M6P receptors are localized predominantly in *trans*-Golgi Network (TGN) and endosomal compartments, whereas only a subset of the receptors are present at the cell surface (Klumperman et al., 1993; Dahms and Hancock, 2002;). Although the exact

mechanisms of enzyme transport have yet to be determined, site directed mutagenesis experiments have shown that binding of clathrin associated proteins to an acidic-cluster-dileucine amino acid (DxxLL) motif within the cytosolic tails of M6P receptors (Fig. 1.4) is required for efficient clathrin-mediated transport of lysosomal enzymes to endosomal compartments (Boker et al., 1997; Ghosh et al., 2003). Previously, interactions between clathrin adaptor protein 1 (AP1) and the dileucine-based sorting signals of M6P receptors, in conjunction with ADP-ribosylation factor, were thought to mediate clathrin-coat assembly on vesicles budding from the TGN (Dell'Angelica et al., 2001, Dahms and Hancock, 2002). Although a role for AP1 in the transport of M6P receptors from TGN-to-endosome has not been ruled out, several recent studies have provided strong evidence that, rather than AP1, it is members of the clathrin-associated Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding (GGA) protein family, which mediates M6P receptor sorting into vesicles budding from the TGN (Fig. 1.4) (Boman et al., 2000; Dell'Angelica et al., 2000). The GGAs, which comprise three members in mammals (GGA1, GGA2 and GGA3) and two members in yeast (Gga1p and Gga2p), are monomeric modular proteins consisting of four domains: an amino-terminal VHS (for VPS27, Hrs, STAM homology) domain, a GAT (for GGA and TOM homology) domain, a connecting hinge segment, and a carboxy-terminal GAE (for γ-adaptin ear homology - a subunit of AP-1) domain (Dell'Angelica et al., 2000; Ghosh et al., 2003). The GAT domain binds ADP-ribosylation factor-guanosine 5V-triphosphate complexes and mediates recruitment of GGAs from the cytosol onto the TGN. The VHS domain interacts specifically with the DxxLL motif in the cytoplasmic tails of the M6P receptors. Mutations in the DxxLL motif impair sorting and decrease M6P receptor binding to the GGAs, indicating that this interaction is critical for sorting at

the TGN. The GAE domain binds a subset of the accessory factors that interact with the ear domain of AP-1, whereas the recruitment of clathrin triskeletons to budding vesicles is most likely mediated through clathrin binding motifs of the hinge and GAE domain. Taken together, these findings suggest that GGAs are sorting proteins that recruit M6P receptors into clathrin coated vesicles at the TGN for their transport to endosomes (Dell'Angelica et al., 2000; Ghosh et al., 2003). By contrast, receptor recycling from endosomes back to the TGN does not seem to be clathrin-mediated (Draper et al., 1990; Iversen et al., 2001). Efficient retrieval of M6P receptors to the TGN appears to involve an interaction between the cytoplasmic tail of the receptor and tail binding proteins. Two such candidate tail binding proteins, phosphofurin acidic cluster sorting protein 1 (PACS-1) and MPR tail interacting protein of 47 kDa (TIP47), have been implicated in receptor recycling. PCAS-1, which binds the carboxy terminal acidic cluster of the M6P/IGF-II receptor, also interacts with AP-1 (Diaz et al, 1998; Wan et al, 1998; Ghosh et al., 2003). Anti-sense mediated depletion of PACS-1 or over expression of a mutant PACS-1 that binds cargo tails but not AP1, results in a shift in M6P/IGF-II receptor distribution away from the perinuclear region towards peripheral endosomal structures, as observed in cells lacking AP-1 (Crump et al., 2001; McKinnon et al., 2001). These findings suggest that PACS-1 may act as a connector between the M6P receptors and AP-1 to facilitate recycling of the receptors from early endosomes to the TGN. A role for TIP47 in M6P receptor retrieval is supported by evidence that antibody-mediated reduction of endogenous TIP47 can inhibit M6P receptor transport from late endosomes to the TGN. Optimal TIP47 function depends on binding to Rab9, a late endosome GTPase that increases the affinity of TIP47 for M6P receptors (Diaz et al., 1998; Carrol et al., 2001; Dell'Angelica et al., 2001). These data indicate that PACS-

1/AP-1 likely mediates receptor recycling from early endosomes, whereas TIP47/Rab9 recycles receptors from late endosomes (Dahms and Hancock, 2002; Ghosh et al., 2003). However, the relative contribution of these two pathways to the total M6P receptor retrieval/trafficking remains to be determined.

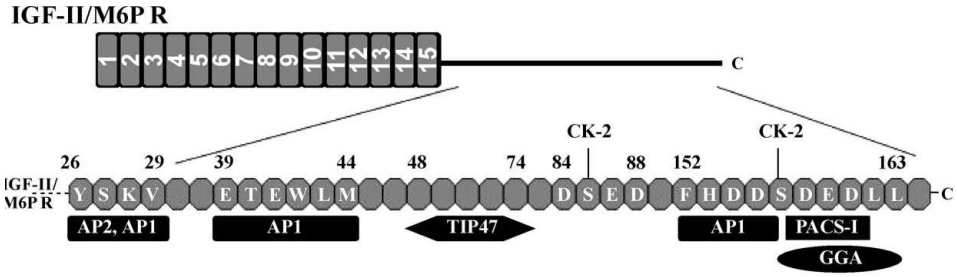


Fig. 1.4 A schematic representation of the amino acid sequence of the M6P/IGF-II receptor cytosolic tail. Amino acid sorting signals (recognized by single letter amino acid code) and their associated transport proteins are identified. Cell surface receptor internalization is mediated by clathrin associated adaptor protein AP-2, while lysosomal enzyme transport is mediated through an interaction with GGA proteins and AP-1. Retrograde receptor trafficking from early endosomes to the Golgi is believed to involve PACS-1/AP-1 binding, while a TIP47/Rab9 interaction recycles receptors from late endosomes. AP-1, adaptor protein 1; AP-2, adaptor protein 2; CK-2, casein kinase 2; GGA protein, Golgi-localized g-ear-containing ADP-ribosylation factor-binding protein; TIP47, tail interacting protein of 47 kDa; PACS-1, phosphofurin acidic cluster sorting protein 1.

1.7b M6P/IGFII receptor and endocytosis of M6P-containing ligands

Cell surface M6P/IGF-II receptors, but not CD-M6P receptors, mediate endocytosis of a variety of M6P-containing ligands for their subsequent clearance or activation. The M6P/IGF-II receptor plays a general role in the recapture of endogenous, newly synthesized lysosomal enzymes which escape sorting at the TGN or that have been actively exported by the CD-M6P receptor (Koster et al., 1994; Hille-Rehfeld, 1995). Endocytosis of lysosomal enzymes by the M6P/IGF-II receptor serves as a mechanism to facilitate degradation of extracellular matrix proteo-glycans or to transfer enzymes from one cell type to another (Roff et al., 1983; Kornfeld, 1992). There is evidence that this receptor also mediates the internalization and subsequent degradation or activation of proliferin (a prolactin-related murine protein) (Lee et al.,

1988), glycosylated human LIF (Blanchard et al., 1999), renin precursor (Saris et al., 2001) and epidermal growth factor receptor (Todderud et al., 1988). The internalization process appears to involve the formation of clathrin coated vesicles in a process mediated by the interaction between clathrin associated adaptor protein AP2 and the single tyrosine-based internalization motif YSKV, located on the cytoplasmic tail of the M6P/IGF-II receptor (Kornfeld, 1992; Dahms and Hancock, 2002).

1.7c The M6P/IGF-II receptor binds some prohormones and may play a role in their processing

In addition to lysosomal enzymes, several other proteins have been demonstrated to possess mannose 6-phosphate and to be capable of binding to the M6P/IGF-II receptor. In general, the lysosomal enzymes possess complex oligosaccharide structures that contain phosphorylated mannose residues, and thereby exhibit distinct binding to the receptors. On the other hand some prohormones possess only one or two mannose 6-phosphates and bind with lower affinity (Fischer et al., 1982; Dong et al., 1990). This may explain the phenomenon whereby the vast majority of lysosomal enzymes are sorted directly from the Golgi apparatus to the lysosome, while the prohormones often escape the cell and are later recaptured. Some examples are pro-transforming growth factor TGF-β, proliferin, growth factors, and thyroglobulin (the precursor to thyroid hormones). The cell surface M6P/IGF-II receptor is also believed to facilitate activation of the TGF-β precursor (Dennis et al., 1991; Ghahary et al., 1999), the proform of a hormone which regulates differentiation and growth of many cell types. The latent pro-TGF-β, one component of which contains M6P residues, is secreted from cells and stored in the extracellular matrix as an inactive precursor complex that can be converted to its active form only after

processing (Munger et al., 1997; Villevalois-Cam et al., 2003). Although TGF-β activation has been reported to be mediated by the matrix glycoprotein thrombospondin-1 (Crawford et al., 1998), several lines of evidence suggest a role for plasmin-mediated activation of TGF-β following its binding to cell surface IGF-II/M6P receptors (Dennis et al., 1991; Ghahary et al., 1999). Moreover, recent data, which demonstrated the ability of plasminogen and the urokinase-type plasminogen activator receptor (uPAR) to bind the M6P/IGF-II receptor at regions distinct from the M6P binding pockets, support a plausible model in which binding of urokinase plasminogen activator to a uPAR that is complexed to the receptor, facilitates conversion of plasminogen to plasmin, which in turn proteolytically activates receptor bound TGF-β precursor (Godar et al., 1999; Ghosh et al., 2003).

Pro-transforming Growth Factor- β

The presence of two phosphorylated oligosaccharides at Asn 82 and 136 on pro-TGFβ1 has recently been determined (Purchio et al., 1988) and it has been demonstrated that this protein binds to the M6/IGF-II receptor in a mannose 6-phosphate inhibitable manner (Kovacina et al., 1989). The presence of a latent, precursor form of TGF-β has previously been suggested (Nilsen-Hamilton et al., 1980; Wakefield et al., 1988 ;). It is of interest that while there are multiple forms of TGFβ (Derynck et al., 1985; Obberghen-Schillng et al., 1987; Marquardt et al., 1987; Dijke et al., 1988) only the first form, TGFβ, has been shown to possess the lysosomal enzyme recognition marker mannose 6-phosphate). The precise role of the TGFs is to play a role in the production of collagen matrix and its interaction with cells (Ignotz et al., 1987; Montesano et al., 1988) and in the control of proliferation, differentiation, and transformation (Sporn et al., 1986; Keski-Oja et al., 1987). The

physiological role of the binding of pro-TGF- I to the M6P/IGF-II receptor is also not known at this time, but it may play a role in the processing of this prohormone.

Proliferin

The next growth factor that possesses a mannose 6-phosphate moiety and can bind to the mannose 6-phosphate receptor is proliferin (Lee et al., 1988). Proliferin is an approximately 25 kDa glycoprotein growth factor which is secreted by mouse placental tissue and a number of growing mouse cell lines (Lee et al., 1988). It is related to the placental lactogens (Lee et al., 1988) and shows a 31 % identity with prolactin. At this time the only receptor identified for this molecule is the M6P/IGFII receptor, which it binds with a high affinity (kd>1-2 nM) which is dependent upon glycosylation and phosphorylation and can be inhibited by mannose 6-phosphate (ki~ l0μM) or anti-M6P/IGF-II receptor antibodies (Lee et al., 1988). The functional significance of the proliferin-receptor interaction is still not fully understood.

Thyroglobulin

The best studied prohormone possessing mannose 6-phosphate recognition marker is thyroglobulin (Yamamoto et al., 1985; Herzog et al., 1987). Thyroglobulin is a large protein which is the precursor to the thyroid hormones T3 and T4. Thyroglobulin is secreted into the thyroid follicle lumen where it is iodinated and concentrated (Herzog et al., 1987). The iodinated thyroglobulin then binds the M6P/IGF-II receptor and is endocytosed. It then arrives in a lysosome population which may be distinct from the cells degradative lysosomes (Selmi et al., 1988). The iodinated thyroglobulin is then processed into the mature thyroid hormones by sequential cleavage by cysteine proteinase I, cathepsin B, and other lysosomal proteases (Dunn et al., 1988). The mature thyroid hormones thus produced can then be released as needed.

1.8. Multi-functional nature of the M6P/IGF-II receptor

1.8a. M6P and human IGF-II interaction

The non-glycosylated 67 amino acid IGF-II peptide is the best-characterized non M6P-containing ligand of the M6P/IGFII receptor (O'Dell et al., 1998; Dahms and Hancock, 2002). Several lines of experimental evidence over the last decade have clearly indicated that IGF-II plays a crucial role in mammalian growth by influencing fetal cell division and differentiation (Ludwig et al., 1995; O'Dell et al., 1998). Interestingly, the growth promoting effects of IGF-II are believed to be mediated by its ability to bind IGF-I and/or insulin receptors and the role of the M6P/IGF-II receptor is to divert excess IGF-II for its degradation in lysosomes. This is supported in part, by experimental data which have shown that i) antibodies against the receptor do not inhibit the mitogenic effect of IGF-II (Kiess et al., 1987), while IGF-I receptor blocking antibodies impair IGF-II action in various cell culture systems (Furlanetto et al., 1987); (ii) viable IGF-II-deficient mice are 40% smaller than their wild type siblings (Baker et al., 1993); and (iii) IGF-II mutants with a weak affinity for the M6P/IGF-II receptor but a high-affinity for the IGF-I receptor induce biological responses (i.e., stimulation of DNA synthesis in BALB/c 3T3 cells and glycogen synthesis in Hep G2 cells) in correlation with their affinity for the IGF-I receptor (Sakano et al., 1991). On the other hand, IGF-II recognition and internalization by the M6P/IGF-II receptor is postulated to be a general mechanism used to modulate circulating levels of IGF-II by targeting it for lysosomal degradation. This is substantiated, at least in part, by gene targeting studies which have shown that mice lacking the M6P/IGF-II receptor exhibit fetal overgrowth, elevated levels of circulating IGF-II and perinatal lethality as a consequence of major cardiac abnormalities (Lau et al., 1994; Wang et al., 1994). Interestingly, this phenotype can be completely

rescued by simultaneous deletion of (knocking out) either the IGF-II peptide or IGF-I receptor gene (Ludwig et al., 1996), thus suggesting that the lethality observed in M6P/IGF-II receptor-deficient mice is caused by an over stimulation of the IGF-I receptor by excess IGF-II. While the function of the M6P/IGF-II receptor in IGF-II clearance is well accepted, its role in mediating any biological actions of the growth factor remains controversial. Several studies, however, indicate that binding of IGF-II to the M6P/IGF-II receptor can induce specific responses, including increased amino acid uptake in muscle cells (Shimizu et al., 1986), glycogen synthesis in hepatoma cells (Hari et al., 1987), exocytosis of insulin from pancreatic cells (Zhang et al., 1997), cell proliferation in K562 erythroleukemia cells (Tally et al., 1987), increased gene expression in spermatocytes (Tsuruta et al., 2000), motility of human rhabdomyosarcoma cells (Minniti et al., 1992), migration of human extravillous trophoblasts (McKinnon et al., 2001), stimulation of Na^+/H^+ exchange and inositol triphosphate production in canine kidney cells (Rogers et al., 1988) and calcium influx (but not cell proliferation) in primed BALB/c3T3 fibroblast cells (Kojima et al., 1988; Matsunaga et al., 1988). Receptor specificity in most cases was confirmed by the use of a rather selective M6P/IGF-II receptor analogue, receptor antibodies which mimic/block IGF-II effects, or evaluating the effects in a system which lacks IGF-I receptors (McKinnon et al., 2001; Minniti et al., 1992). Given that the cytoplasmic tail of the M6P/IGF-II receptor lacks a kinase domain, the intracellular mechanisms by which the receptor can mediate such biological effects remain unclear. However, a number of studies in cell-free experimental systems and a few studies in living cells have provided evidence for an interaction of the M6P/IGF-II receptor with heteromeric G proteins (Ikezu et al, 1995; Minniti et al, 1992). By comparing the sequence of the human M6P/IGF-II receptor with that of mastoparan,

a small peptide in wasp venom that can directly activate Gi and Go proteins (Higashijima et al., 1990), it has been shown that a 14 residue amino acid residue (Arg2410–Lys2423) in the cytoplasmic region of the M6P/IGF-II receptor can mediate Gi_{α} activation. This is supported by evidence that adenylate cyclase activity was inhibited by IGF-II in COS cells transfected with constitutively activated Gi_{α} (Nishimoto et al., 1990; Okamoto et al., 1990; 1991) and wild-type M6P/IGF-II receptor cDNAs, but not with M6P/IGF-II receptors lacking Arg2410–Lys2423. Furthermore, homology was noted between the C-terminal Ser2424–Ile2451 region of the M6P/IGF-II receptor and part of the pleckstrin homology domain of several proteins that bind $G_{\beta\gamma}$ and inhibit its stimulatory action on adenylate cyclase activity (Ikezu et al., 1995). At the functional level, there is evidence to suggest that IGF-II, acting via a Gi protein, can stimulate Ca^{+2} influx in 3T3 and CHO cells (Kojima et al., 1988; Matsunaga et al., 1988), increased exocytosis of insulin from the pancreatic cells (Zhang et al., 1997) and promote migration of extravillous trophoblast cells (McKinnon et al., 2001). Additional findings have shown that M6P/IGF-II receptor-activated G protein can lead to PKC-induced phosphorylation of intracellular proteins (Zhang et al., 1997), stimulation of MAP kinase pathway and/or decrease in adenylate cyclase activity (McKinnon et al., 2001). These results, taken together, suggest that the M6P/IGF-II receptor may mediate certain biological effects of IGF-II, most likely via activation of a G-protein coupled pathway. However, given the evidence that M6P/IGF-II receptor, under certain conditions, failed to interact with G protein or to couple Giα (Korner et al., 1995), the overall significance of the receptor - G protein interactions under in vitro conditions and its relevance to normal physiology are a matter of speculation.

1.8b. M6P/IGF-II receptor and cancer

The ability of the receptor to modulate levels of the mitogen IGF-II (O'Dell et al., 1998), to facilitate activation of the growth inhibitor TGF-β (Dennis et al., 1991; Ghahary et.al, 1999) and to regulate targeting of lysosomal enzymes to lysosomes (Hille-Rehfeld, 1995; Kornfeld, 1992), suggests that the receptor could act as a tumour suppressor. This is supported by data which showed that over expression of the receptor acts as a growth inhibitor under both *in vitro* and *in vivo* conditions (O'Gorman et al., 2002), whereas loss of receptor function is associated with progression of tumorigenesis (Oates et al., 1998; DaCosta et al., 2000). Frequent loss of heterozygosity at the M6P/IGF-II receptor locus has been reported for a variety of cancers including liver (De Souza et al., 1995; Yamada, et al., 1997), breast , ovarian (Hankins et al., 1996; Chappell et al., 1997; Rey et al., 2000) and lung (Kong et al., 2000). In some of these cases, somatic mutations in the remaining parts of the receptor allele have been identified, the majority of which disrupt M6P and/or IGF-II binding properties (Byrd et al., 1999; Devi et al., 1999), While only a single point mutation (P2379T) in the cytosolic tail has been identified, nine human cancer-associated mis-sense mutations of the extracytoplasmic region of the MPR/IGFII have been reported: two are located in M6P recognition domain 9 (C1262S, G1296R), three are in domain 10 (Q1445H, G1449V, G1464E), and four are in IGF-II binding domain 11 (G1564R, I1572T, A1618T, and G1619R). Significantly, with the exception of G1464E, all of the missense mutations (C1262S, Q1445H, G1449V, G1464E, and I1572T) that have been characterized to date result in receptors with altered M6P and/or IGF-II binding properties, supporting the hypothesis that loss of normal M6P/IGF-II function contributes to carcinogenesis. However, to gain new insights further functional studies need to be carried out.

The M6P/IGF-II receptor has also been reported to bind retinoic acid to induce changes in cell shape, growth inhibition and apoptosis (Kang et al., 1997; 1999). The ability of the receptor to recognize many functionally distinct ligands illustrates not only the multifunctional role of the receptor, but also raises the possibility of its involvement in a myriad of important physiological functions.

1.9. MPRs in the invertebrates and an evolutionary perspective.

1.9a. MPRs in the invertebrates

Although the MPR proteins clearly have a major role in lysosomal enzyme trafficking in the vertebrate cells, their role in invertebrate systems is not well defined. Lysosomal enzymes are successfully targeted in lower eukaryotes such as *Saccharomyces*, *Trypanosoma*, and *Dictyostelium*, without the aid of identifiable MPRs. Both types of MPRs are present in the invertebrates such as the molluscs, (Siva Kumar and von Figura, 2002) but they are not well characterized, and thus, we do not yet know how the MPR genes have evolved. Recently, a *Drosophila* protein (lysosomal enzyme receptor protein, LERP) that is structurally and functionally related to the mammalian CI-MPR was identified (Dennes et al., 2005). LERP mediates lysosomal enzyme targeting and rescues the missorting of lysosomal enzymes that occurs in MPR-deficient mammalian cells. Interestingly, however, the residues that are involved in M6P recognition in mammalian MPRs are not conserved in the LERP and it does not bind to the multimeric M6P ligand phosphomannan. The nature of the interaction between LERP and mammalian lysosomal enzymes has not yet been elucidated. Above the molluscs in the echinodermates in this study we also identified the receptors and extensively characterized the MPR 46 protein. The slime mold *Dictyostelium discoideum* produces a novel methyl-phosphomannose sequence on some of its lysosomal

enzymes that can be recognized in vitro by the mammalian CI-MPR (not the CD-MPR). However, despite the presence of a GlcNAc-Phospho transferase that recognizes α1–2-linked mannose residues, no receptor for the phosphorylated mannose residues has been found in these organisms. Notably, although this phosphotransferase does not show the specific recognition of lysosomal hydrolases as seen with the mammalian enzyme, it produces another transferase that selectively adds GlcNAc-1-phosphate to serine residues. In contrast to this situation, the protozoan *Acanthamoeba* produces a GlcNAc-phospho transferase that does show specific recognition of lysosomal enzymes (Gabel et al., 1984; Couso et al., 1986). Although some of these organisms show evidence for an "uncovering" enzyme, no definable MPR has yet been found.

1.10 An evolutionary perspective and scope of the present study

According to Darwin's theory of evolution (Darwin et al., 1859), natural selection will serve to retain favourable traits and elimate unfavourable ones. For this reason we generally assume that a conserved function or sequence of a protein in different organisms is important to that organism. With respect to the M6P/IGF-II receptor protein that has been shown to bind not only lysosomal enzymes but also IGF-II, we would expect the IGF-II binding capacity to play an important role in mammals. Indeed by experimentation, it has been demonstrated that the primary purpose of the binding of IGF-II by the M6P/IGF-II receptor is to degrade IGF-II and this activity is essential for normal mammalian development. The acquisition of an IGF-II binding site by the receptor appears to have occurred after the divergence of marsupial and placental mammals from their common ancestor with egg-laying mammals, and it has been suggested that this acquisition was a major factor in driving the evolution of an imprinted M6P/IGF-II receptor in some mammals (Killian et al., 2000). However,

a high affinity IGF-II binding site has been described in the receptor of a teleost fish, early vertebrate, (Mendez et al., 2001), raising the possibility that IGF-II binding was an ancestral property of the receptor and also it may reflect an evolutionarily ancient aspect of lysosome biogenesis that predates the M6P-dependent trafficking of lysosomal enzymes seen in present-day mammals. It also raises the question of how, and when, the involvement of the M6P-specific system in lysosome biogenesis evolved. Moreover, the homologies exhibited by the domain structures and ligand-binding activities of some of the vertebrate MPRs raise questions about their phylogenetic origin, evolutionary history, functional significance *in vivo* and the specific evolutionary divergence point at which the complete MPR system came into existence. Answers to these questions can only be known by carrying out detailed comparative studies of the receptors from different non-mammalian vertebrates, invertebrates using animal models as well as available specific cell types.

The present study was started to get some answers for the above questions, with special emphasis to define the multifunctional role of the M6P/IGF-II receptor in the non-mammalian vertebrate species (chicken), clone, characterize the reptilian IGF-II domain structure to understand its functions in the vertebrates, and to purify and characterize the invertebrate receptors (starfish). The details of these have been described in this thesis.

Multi functional nature of non-mammalian M6P/IGF-II receptor

The following sentence gives some details about the multifunctional nature of the known M6P/IGF-II receptors. Among the two MPRs only the mammalian MPR 300 has so far been shown to be a multifunctional protein which, in addition to binding mannose 6-phosphate containing lysosomal enzymes, also binds insulin-like growth factor-II (IGF-II), other ligands such as retinoic acid and thyroglobulin. The receptor has been classed as the M6P/IGF-II receptor. In humans, it has been shown that the M6P/IGF-II receptor plays an important role in controlling the extracellular level of the IGF-II by mediating its binding at the cell surface and delivery to lysosomes. The luminal ligand binding domain of MPR 300 contains 15 internal repeats (cassettes), which are homologous to each other and to the single luminal domain of MPR 46. It is therefore, of interest to study the evolution and functions of these receptor proteins in order to analyze their biochemical and ligand binding properties. MPR 300 proteins purified from a number of species such as, human, rat, bovine, and opossum exhibited IGF-II binding abilities and the IGF-II binding domain in mammals has been localized to repeat 11 of this multifunctional receptor. The biochemical and immunological properties of the purified MPR 300 protein from other animal species such as goat, chicken, garden lizard, fish (non-mammals) and *unio* (invertebrate) resemble those of the mammalian receptor. Further a partial cDNA clone for the fish MPR 300 has revealed that the receptors in the vertebrates contain conserved cassette structures in the luminal domain and the mannose 6-phosphate binding region in the third cassette exhibits extensive sequence homology among all the vertebrates, suggesting that this protein is evolutionarily conserved (Udaya Lakshmi et al., 2000). However, in a recent study it has been shown that the MPR 300 from the early non-mammalian vertebrate, fish binds human IGF-II under specific

conditions (Mendez et al., 2001). This recent observation led us to believe that possibly the IGF-II binding property and the multifunctional nature of the MPR 300 may also be conserved in the other non-mammalian vertebrates such as chicken and reptiles.

Further details about the IGF-II binding of the receptor have been described under Part-II of this chapter.

In the present study the following objectives are studied in detail

Part – I describes about the analysis of the functions of the M6P/IGF-II receptor in chicken for its ability to bind thyroglobulin.

Part – II describes the binding of the reptilian receptor to human IGF-II, cloning and characterization of the IGF-II domain.

SECTION-A

INTRODUCTION

Two distinct but homologous Mannose 6-phosphate receptors (designated as the MPR 300 (M6P/IGF-IIR, Mr 300 kDa) and MPR 46, (Mr 46 kDa) are involved in transporting lysosomal enzymes from *trans-golgi* to the prelysosomal compartment. These proteins are conserved in the vertebrates from fish to mammals. The mammalian M6P/IGF-IIR has been shown to be a multifunctional protein, which in addition to sorting lysosomal enzymes intracellularly, also endocytoses the lysosomal enzymes, shows specific interaction with human IGF-II and thyroglobulin (Tg). The porcine M6P/IGF-II receptor has been demonstrated to bind the porcine thyroglobulin in a mannose 6-phosphate dependent manner (Herzog et al., 1987). The multiple cassette structures present in the mammalian protein and the ability of the mammalian protein to bind variety of ligands raised a question whether the multifunctional nature of the protein is also conserved in evolution, particularly among the vertebrates, where the M6P/IGF-II receptor has been characterized. The chicken embryonic fibroblast (CEF) cell M6P/IGFII-R is biochemically and immunologically similar to the mammalian protein and has so far been shown to sort lysosomal enzymes and binds human IGF-II (Suresh et al., 2006). Therefore, we wanted to analyze if this protein can also bind additional ligands such as the thyroglobulin. Due to the ready availability of the bovine thyroglobulin, all our studies were carried out with this protein. We used the purified chicken liver M6P/IGF-II receptor as well as the CEF cells in our experiments to study the binding of the receptor to thyroglobulin.

SECTION-B

MATERIALS

O-phosphonomannan was a generous gift from Dr.M.E.Slodki, USDA, and Peoria, IL, USA. Affinity purified antibody to the goat MPR 300 protein was as described (Suresh et al., 2002). Mannose 6-phosphate (M6P), human IGF-II, bovine thyroglobulin (Tg), Tg monoclonal antibody, DMEM, trypsin-EDTA, penicillin-streptomycin and FITC were purchased from Sigma. TRITC coupled anti-mouse IgG was purchased from Bangalore Genei, India. FBS was purchased from JRH Bioscience and radioactive iodine $Na^{125}I$ from MP Biomedical USA. Fresh chicken liver tissue was purchased from the local slaughter house and carried on ice to the laboratory and used to purify the MPR 300 protein.

METHODS

Cell culture

Chicken embryonic fibroblast (CEF) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum. Cells were grown in standard flasks as well as in 3cm petri plates in an incubator at 37°C in an atmosphere containing 5% CO2 as described (Matzner et al., 1996).

Extraction of membrane proteins from CEF cells

Confluent monolayers grown in 90 cm petri plates were scraped with the help of a cell scraper, and the cell pellet was collected by centrifugation in a Biofuge stratos centrifuge, using a microliter rotor at 2991 × g for 10 min. The cells were suspended in 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge, using a

fixed angle 80Ti rotor at 161,280 × g for 35 min. The pellet obtained at this step was dissolved in 50 mM imidazole-HCl buffer pH 7.0 containing 0.5% Triton X-100, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and re-centrifuged as described above. The MPR 300 protein from this membrane extract as well as from the chicken liver membrane extract was purified to homogeneity by phosphomannan affinity chromatography (PM gel) as described (Suresh et al., 2006).

Protein assay

Protein concentrations were determined using BCA reagent following manufacturer's instructions. BSA was used as the standard.

In vitro iodination

Thyroglobulin (50 µg), purified CEF cell MPR 300 protein (10-20 µg) were radio iodinated using 150 µci of [^{125}I] NaI as described (Suresh et al.,2006) to a specific activity of 1200–2500 cpm per ng protein. [^{125}I] Tg was used for quantitation of Tg-binding and internalization studies on CEF cells and radiolabeled MPR 300 was used for affinity chromatography on Tg-Sepharose gel.

Affinity chromatography on Tg-Sepharose

Tg-Sepharose gel and BSA coupled to affigel-10 were equilibrated at 4ºC with 50 mM Tris-HCl, 0.9% NaCl, 0.1% BSA, 5 mM $CaCl_2$ pH 7.4 containing 0.05% Triton X-100 (buffer A) as described (Lemansky et al., 1992) In brief, ^{125} I, radiolabeled CEF M6P/IGFIIR 300 (10, 00,000 cpm) in buffer A (100 µl), was loaded on to the Tg affinity gel (200 µl) or onto the BSA gel (200 µl) at a flow rate of 2.5 ml/min. Unbound proteins were removed by washing with 10 volumes of wash buffer (buffer A). Bound proteins were eluted with buffer A containing 5 mM glucose 6-phosphate

followed by 5 mM mannose 6-phosphate. Column fractions were TCA precipitated, separated by SDS-PAGE and the bands visualized by autoradiography.

Quantitation of Tg binding and internalization [Binding of [^{125}I] Tg to CEF cells]

Radio iodinated Tg was diluted in buffer A to give final concentrations ranging from 8 to 86 nM. Cells were grown in 12 well culture plates, to 80-85 % confluency and incubated with 10 different concentrations (8 to 86 nM) of ^{125}I-Tg for 90 min at 4^{0}C in binding buffer (buffer A without TritonX100) in the presence of 2 mM mannose [to avoid interference with the possible binding of Tg by its mannose residues]. In a separate experiment, non-specific binding was determined in the presence of 2 mg/ml non-radioactive Tg. After the incubation, the cells were washed in PBS containing 1% BSA five times, and lysed with lysis buffer (1% Triton X- 100, 50 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, 0.02% NaN_3 supplemented with 1 mM PMSF, and 1 mM EDTA as protease inhibitors on ice for 30 min. The cell suspension was centrifuged at 100 *x g* for 2 min. The supernatant was discarded and pellets were counted in a gamma counter, and the amount of bound Tg was calculated and normalized to the concentration of the membrane protein. Analysis was performed in duplicates and the average values presented. Saturation and scatchard plot analysis were carried out using the details in (**www.graphpad.com**).

Binding and internalization of [^{125} I] Tg by CEF cells

The binding and internalization of ^{125}I-Tg in CEF cells was compared by preincubating the cells with mannose 6-phosphate (5 mM), glucose 6-phosphate (5 mM), goat MPR 300 IgG (10 µg), unlabeled Tg (200 nM), rabbit IgG (5 µg) and human IGF-II (2 µg). Binding analysis was carried out as described above. For internalization, cells were grown to 80-85 % confluency in 6 well culture plates. The cells were rinsed five times with DMEM containing 20 mM HEPES to remove the

residual serum. [^{125} I] Tg (5, 00,000 cpm) was then added to the cells in 1 ml of serum-free medium and incubated for 30 min at 37^{0}C. At the end of incubation the cells were washed six times with serum-free medium, three times with medium containing 1 mg/ml bovine serum albumin and five times with phosphate-buffered saline. The volume of each wash was 1 ml. After washing the cells, they were incubated with 0.5 ml of lysis buffer as above. Radioactivity in the lysate was measured in a gamma counter.

Treatment of thyroglobulin with Endo H, PNGase F, alkaline phosphatase and its effect on binding and internalization

For glycosidase (Endo H, PNGase F) treatment, [^{125}I] thyroglobulin (5,00,000 cpm) was denatured by boiling with SDS and incubated for 12 hr at 37^{0}C in incubation buffer containing 10 micro units/ µl Endo H and 20 micro units/ µl PNGase F (Sigma) (pH 7.0). For phosphatase treatment [^{125}I] Tg was incubated in 0.15 M NaCl and 0.01 M Tris-HCl buffer pH 8.0, 50 milliunits/µl alkaline phosphatase (Sigma), for 1 hr at 37^{0}C, and then diluted to 1 ml with ice-cold minimal essential medium with 3 mM glutamine or in binding buffer. Effect of these treatments on the binding and internalization of Tg to CEF cells was studied as described above.

Ligand blotting

The membrane proteins extracted from the CEF cells were separated on a 7.5% SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% BSA in PBS the membrane was probed with radio iodinated [^{125}I] thyroglobulin (5, 00,000cpm) in PBS containing 1% BSA for 1hr at room temperature. The membrane was extensively washed with 0.05% Tween 20 in PBS, dried and the bands visualized by autoradiography.

Far Western blotting analysis of enzyme treated and native thyroglobulin

Thyroglobulin (unlabeled) was treated with EndoH, PNGase F and alkaline phosphatase as described above. The enzymes treated as well as the native Tg were electrophoresed and transferred to a polyvinyl-dene-difluoride membrane. After blocking, the membrane was incubated with 50 µg of purified CEF cell M6P/IGFIIR, followed by incubation with goat anti-MPR 300 antibodies (total IgG prepared from the antiserum, 1:1000 dilution) followed by horseradish peroxidase-conjugated anti-rabbit IgG. Detection was done using ECL reagent.

Endocytosis of [^{125}I] thyroglobulin by CEF cells and immunoprecipitation

CEF cells were grown in two 6 cm culture plates one without and one with 5 mM M6P. The plates were rinsed as described above. Iodinated [^{125}I] Tg (5,00,000cpm) was then added to both the plates in 1 ml of serum-free medium and the cells were incubated for 6 hr at 37^{0}C (Lemansky et al., 1992). After removal of the medium, cells were incubated for 3 hr with a chase medium containing 4 mg/ml unlabeled thyroglobulin. At the end of incubation the cells were washed as described above. The cells were lysed by treating with 0.5 ml of 0.05% Triton X-100 containing 0.02% EDTA at 25^{0}C for 5 min and the lysate was transferred into 1.5 ml tubes. The radioactivity in the lysate was measured as above. Thyroglobulin was immunoprecipitated from both the lysates by incubating with mouse monoclonal antibody against Tg (1:200). The immunoprecipitates were finally separated on a 7.5% SDS-PAGE under non-reducing conditions and the bands visualized by autoradiography.

In vitro fluorochromation of Tg

Bovine Tg, 5 mg was incubated with 550 µl borate buffer (50 mM, pH 9.0) and 100 µl fluorochrome solution of fluoresce-inisothiocyanate [5 mg FITC dissolved in one ml

of DMSO] overnight at 4^0C. Free fluorochrome was removed by desalting using a Sephadex G-25 gel as described above.

Immunofluorescence microscopy

Cells were grown on cover glass slides. For Tg binding, the CEF cells were fixed in 4% formaldehyde followed by blocking with 5% BSA in PBS before incubation with 200 nM Tg in binding buffer (buffer A without TritonX100) for 90 min at 4^0C. After incubation, cells were immunolabeled with monoclonal mouse anti-Tg (1:200) for 1hr. Washing was done as described above, and TRITC coupled anti-mouse IgG (1:1000) was used as the secondary antibody. For internalization of Tg, cells were incubated for 30 min with serum free DMEM supplemented with 200 nM FITC-Tg. After incubation, cells were washed with PBS containing 1% BSA (five times, for 2 min). Then cells were fixed with 4% formaldehyde, and observed under confocal microscopy. For detection of the Tg and arylsulfatase A in the endosomal compartments, FITC-Tg (200 nM) was internalized on CEF cells at 37^0C for 30 min and then cells were fixed with 4% Para formaldehyde, permiablized with 0.2% Triton X-100, after blocking, cells were incubated with the Human arylsulfatase A antibody and detected using Alexa fluor-594 labeled secondary antibody. For the colocalization of Tg with M6P/IGF-IIR, cells were incubated with FITC-Tg at 37^0C for 30 min to allow internalization and permeabilized as described above, washed and incubated with goat MPR 300 IgG (10 µg), followed by TRITC labeled secondary antibody (1:1000).

SECTION-C

RESULTS

Tg-Sepharose affinity chromatography

The MPR 300 protein from CEF cells and chicken liver was purified to homogeneity (data not shown) and radio iodinated as described under methods. These proteins were separately applied on to Tg-Sepharose gel. (BSA–affigel-10 matrix prepared in the laboratory was used as a control gel). The gels were processed as described under methods and the column fractions and eluates were analysed by SDS-PAGE. The CEF cell M6P/IGFIIR and the chicken liver MPR 300 were both bound on Tg-Sepharose gel and could be specifically eluted using 5 mM M6P (Fig 2.1A and B). [Under similar conditions the goat purified and radio iodinated MPR 300 was bound to the gel, data not shown]. When the iodinated receptors were applied on BSA-affigel, all the radioactivity could be recovered in the unbound fraction suggesting no binding of the receptors to BSA (data not shown).

Upon increasing the incubation time with fixed concentration of the radiolabeled thyroglobulin with the CEF cells, there was an increase in the number of counts bound to the cell surface or internalized until about 90 min beyond which there is no change in the number of counts bound to the cell surface. Similar results were obtained with respect to the internalized counts suggesting that the Tg was bound and internalized into the cells (Fig 2.2).

Characterization of Tg binding to Chicken embryonic cells

To characterize the Tg binding to the CEF cells, these were incubated with radio iodinated Tg with or without 3 µM non-radioactive Tg at 4^0C. In the absence of non-radioactive Tg, the amount of cell-bound [^{125}I] Tg increased with increasing amounts of free radiolabled Tg exhibiting saturation kinetics (Fig 2.3A open circles). In the

presence of non-radioactive Tg, the cell-bound radioactivity was lower, and increased in a linear fashion (Fig 2.3A, triangles). The specific binding of [^{125}I] Tg to the surface of CEF cells was calculated (Fig 2.3B) by subtracting non-specific binding (Fig 2.3A, triangles) from total binding (Fig 2.3A, open circles). Saturation of specific Tg binding to CEF cells was reached at 66 nM of Tg. Scatchard analysis revealed a dissociation constant of 33 nM (Fig 2.3C).

Binding and internalization of Tg in presence of various ligands, and effect of glycosidases and alkaline phosphatase treatment of Tg on binding and internalization.

When radiolabeled Tg was incubated with the CEF cells at 4ºC, the binding of Tg to the cell surface was observed. This binding was inhibited by mannose 6-phosphate, and MPR 300 IgG, while glucose 6-phosphate, rabbit IgG and human IGF-II had no effect. Similar observations were seen for the internalization of the radiolabeled Tg. Additionally, deglycosylation and dephosphorylation of the bovine Tg was done as described under methods and the effect of these treatments on binding and internalization in CEF cells was also studied. The data from these experiments reveals that treatment with glycosidase enzymes abolished almost 90% of the binding and internalization of radiolabeled Tg while treatment with alkaline phosphatase suggested that the uptake was inhibited to 95%. These results are presented in Figure 2.4 A and B.

M6P/IGFIIR interacts with thyroglobulin in vitro

The binding of the radiolabeled Tg to the CEF cell MPR 300 was analysed in a ligand blot experiment. From Figure 2.5A, it is apparent that a band could be visualized in the region of the MPR 300 protein suggesting binding of the iodinated Tg to the CEF cell MPR 300 protein. The binding capacity of Endo H, PNGase F and

alkaline phosphatase treated Tg to MPR 300 protein was demonstrated *invitro* using the purified M6P/IGFIIR as a probe in a far Western blot analysis. Tg untreated with enzymes, showed clear recognition by the receptor (Fig 2.5B, lane 1). Treatment of the Tg with Endo H, PNGase F or with phosphatase completely abolished the binding (Figure 2.5B, lanes 2, 3 and 4) suggesting the importance of M6P moieties for specific binding.

Degradation of Tg in chicken embryonic fibroblast cells

Intracellular degradation of Tg was analysed by incubation of CEF cells with radio iodinated Tg at 37^0C with or without 5 mM M6P as described under methods. The cell lysates were immunoprecipitated using monoclonal antibody to Tg and analysed by SDS-PAGE and autoradiography. From the results (Fig 2.5C, lane 1) it is suggestive that the radiolabeled Tg was internalized and fragmented in the cells possibly by proteolysis. However the presence of M6P abolished its binding to the receptor and could not be internalized for further proteolytic breakdown (Fig 2.5C, lane 2).

Fluorescence microscopy analysis

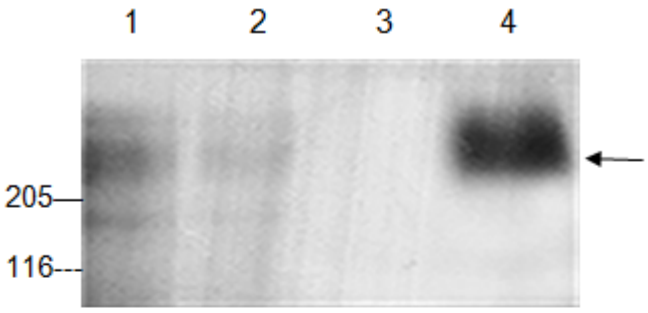
CEF cells were incubated with Tg to study binding as well as internalization using fluorescence microscopy. Incubation of CEF cells with Tg at 4^0C followed by immunolabeling, revealed a patched staining pattern along the entire cell surface (Fig 2.6A). Incubation with FITC-labeled Tg for 30 min at 37^0C, showed fluorescence of Tg in endocytic vesicles. Tg containing vesicles were distributed throughout the cytoplasm of CEF cells. For identification of those vesicles as early or late endocytic compartments, CEF cells were immunolabeled with antibodies against the lysosomal enzyme arylsulfatase A (Fig 2.6B). It is well established in mammals by morphology and by the immunocytochemical detection of lysosomal

enzyme arylsulfatase A marker protein, these Tg-containing vesicles were identified as prelysosomal or early endosomes. The results indicated that Tg was bound to the plasma membrane of CEF cells at 4^0C, and was internalized when incubations were done at 37^0C.To confirm that M6P/IGF-IIR and endocytosed Tg are colocalized and present in the early or late endosomal compartments, cells were immunolabeled with antibodies against M6P/IGF-IIR and examined by immunofluorescence microscopy. As shown in Fig 2.6C, colocalization is distinctly observed and M6P/IGF-IIR is located together with Tg in vesicles surrounding the nucleus corresponding to late endosomes.

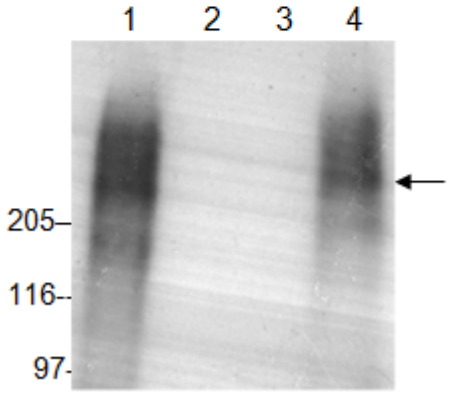
Figure: 2.1 Affinity chromatography on Tg-Sepharose gel. A) Purified and radio iodinated CEF MPR 300. 7.5% SDS-PAGE analysis. Lane 1, unbound fraction, lane 2, wash, lane 3, glucose 6-phosphate eluate and lane 4, mannose 6-phosphate eluate (specific elution).

B). Purified and radio iodinated chicken liver MPR 300. 7.5% SDS-PAGE analysis. Lane 1 unbound fraction [It is seen from the figure that some the radioactivity applied was found, which could be due to overloading on the affinity matrix] lane 2 wash, lane 3, glucose 6-phosphate eluate and lane 4 mannose 6-phosphate eluate.

Figure: 2.2 Time course for binding and internalization of [^{125}I-Tg] by CEF cells. CEF cells were grown in 12 well culture plates and incubated with 1, 00,000 cpm [^{125} I-Tg] in binding buffer at different time intervals at 4^0C as well as 37^0C, washings and lysis was done as described in methods. Solid line and closed circles are surface-associated, dotted line and open circles are Internalized [^{125} I-Tg] respectively.



(A)



(B)

Figure 2.1

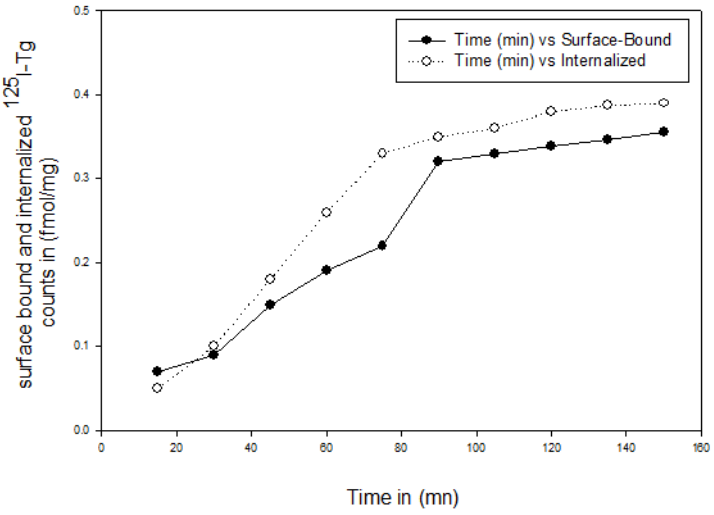


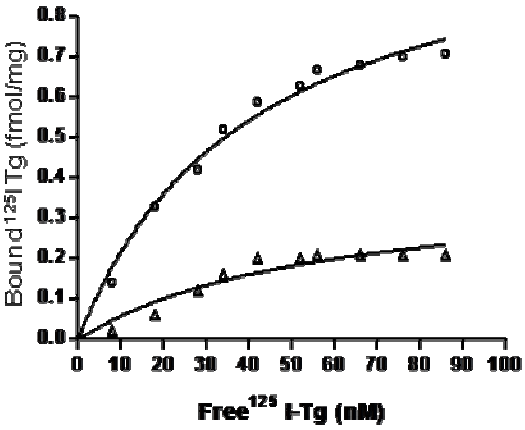
Figure 2.2

Figure 2.3: Saturation binding assay of Tg binding on Chicken embryonic fibroblast cells. Cells were incubated with increasing amounts of radio iodinated Tg at 4^0C.

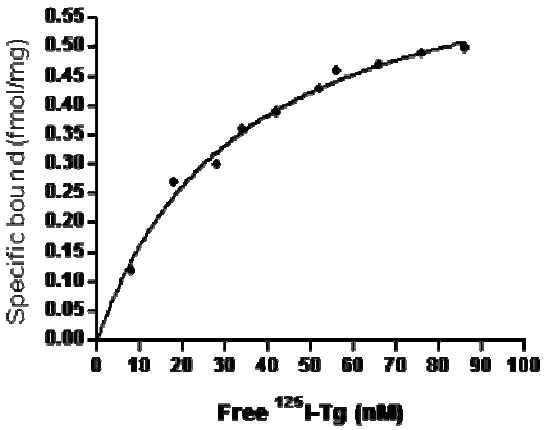
A). In the presence (*triangles*) or absence (*circles*) of 2 mg/ml non-radio iodinated Tg. Non-specific binding (**A**, *triangles*) was subtracted from total binding (**A**, *circles*) to give specific binding of Tg to chicken embryonic fibroblast cells (**B**, *filled circles*).

B) The amount of bound Tg increased with increasing amounts of freeTg, and was saturable at 66 nM Tg.

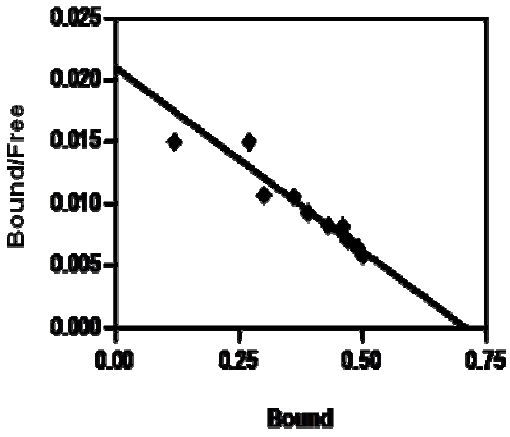
C). Scatchard plot analysis demonstrated a dissociation constant of 33 $k_{d.}$



(A)



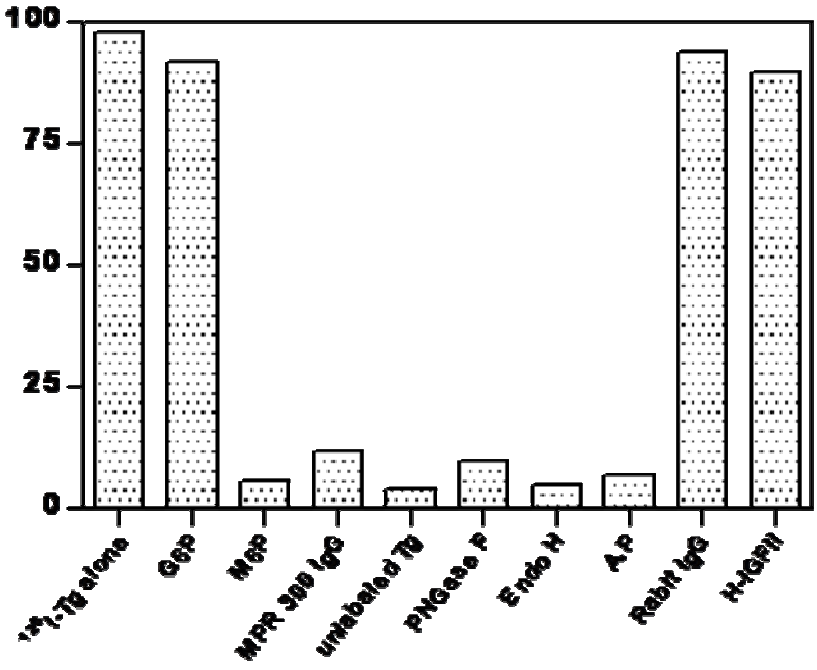
(B)



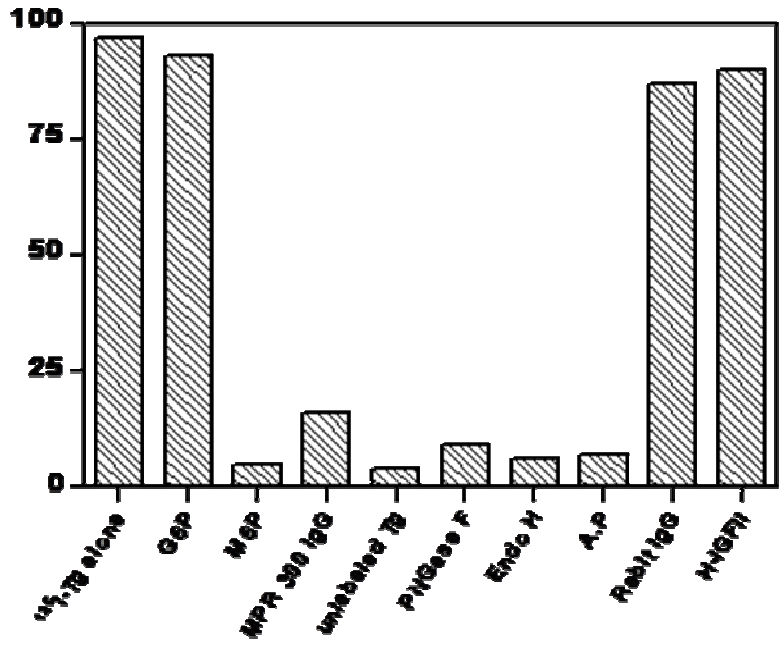
(C)

Figure 2.3

Figure 2.4: Binding and internalization of [^{125}ITg] by CEF cells. Cells were incubated with [^{125}I-Tg] without any ligand and with different ligands shown in as described under methods. The % of radioactivity bound (panel A) and internalized (panel B) was analysed.


100
75
50
25
0
GcP
MgP
MPR 300 IgG
unlabeled Tg
PNGase F
Endo H
A.P
Rabbit IgG
H-IGPR

(A)


100
75
50
25
0
GcP
MgP
MPR 300 IgG
unlabeled Tg
PNGase F
Endo H
A.P
Rabbit IgG
H-IGPR

(B)

Figure 2.4

Figure: 2.5 *In vitro* binding of Tg to CEF MPR 300.

A). Ligand blotting**-** CEF membrane proteins (25 µg) were separated on 7.5% SDS-PAGE, transferred to PVDF membrane and incubated with 5, 00,000 cpm of radio iodinated Tg, washed and exposed to X-ray film. Protein band detected by autoradiography.

B). Binding of native and enzymes treated thyroglobulin with purified CEF cell M6P/IGFIIR was analyzed by far Western blotting using M6P/IGFIIR as a probe Thyroglobulin (20 µg) was incubated with buffer alone (lane 1), Endo H (lane 2), PNGase F (lane 3) and alkaline phosphatase treated (lane 4).

C). Mannose 6-phosphate dependent endocytosis of thyroglobulin by CEF cells. Thyroglobulin was incubated with CEF cells in the presence and absence of 5 mM mannose 6-phosphate. From these cell lysates, Tg was immunoprecipitated, analyzed by 7.5% SDS-PAGE and protein bands detected by fluorography. Lane 1, proteolysis of Tg can be seen in the absence of 5 mM mannose 6-phosphate while no such effect was seen in presence of 5 mM mannose 6-phosphate (lane 2).

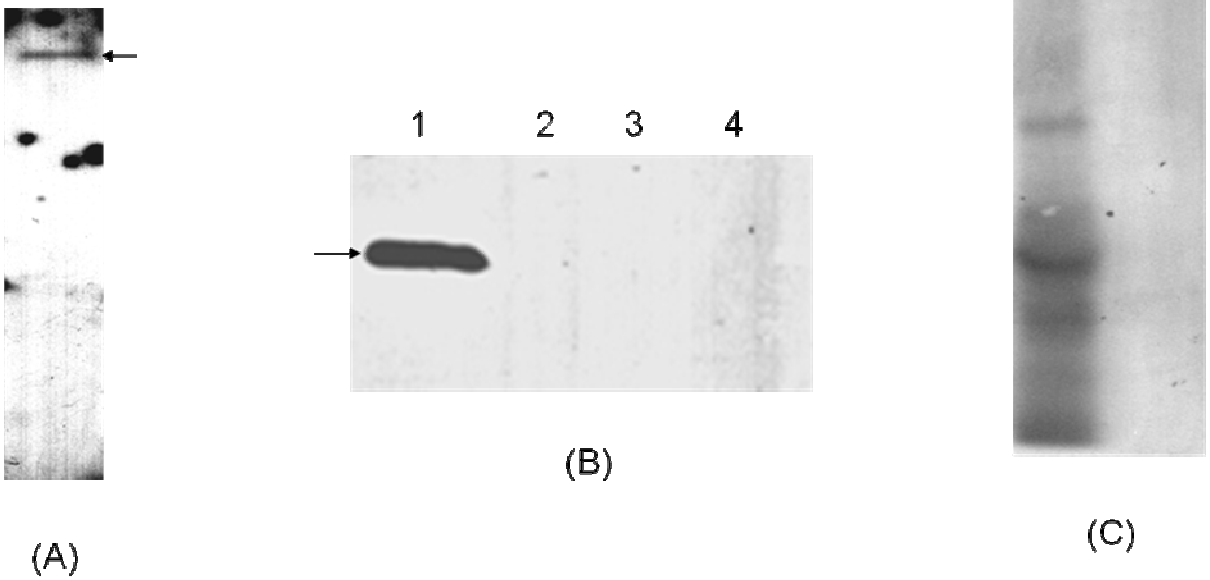


Figure 2.5

Figure 2.6: a) Binding of Tg by CEF cells. Cells were incubated with Tg at 4^0C, and were immunolabeled with monoclonal antibodies against Tg or mouse IgG (Isotopic control). Tg binding was seen along the cell surface (arrowheads). (A-D, see details in figure, (*Bar 11.7 µm*).

b) Endocytosis of FITC-Tg and localization of Arylsulafatase A in the endocytic compartments. (A) FITC Tg, **(B)** localization of arylsulfatase A and (C) merge of (A and B) (see details in figure A-D, (*Bars 11.32µm*)

c) Colocalization of M6P/IGFIIR and FITC-Tg within endocytic compartments of CEF cells. (A) Internalization of FITC labeled Tg at 37^0C **(B)** After fixation, cells were immunolabeled with polyclonal anti-Goat MPR 300 antibody and TRITC-labeled secondary antibody. **(C)** Co-localization of M6P/IGFIIR **(A)** and FITC-Tg **(B)** within early or late endosomal compartments of CEF cells. **(C)** Merge of **A** and **B**. (See details figure **A-D**) (*Bars, 11.5µm*).

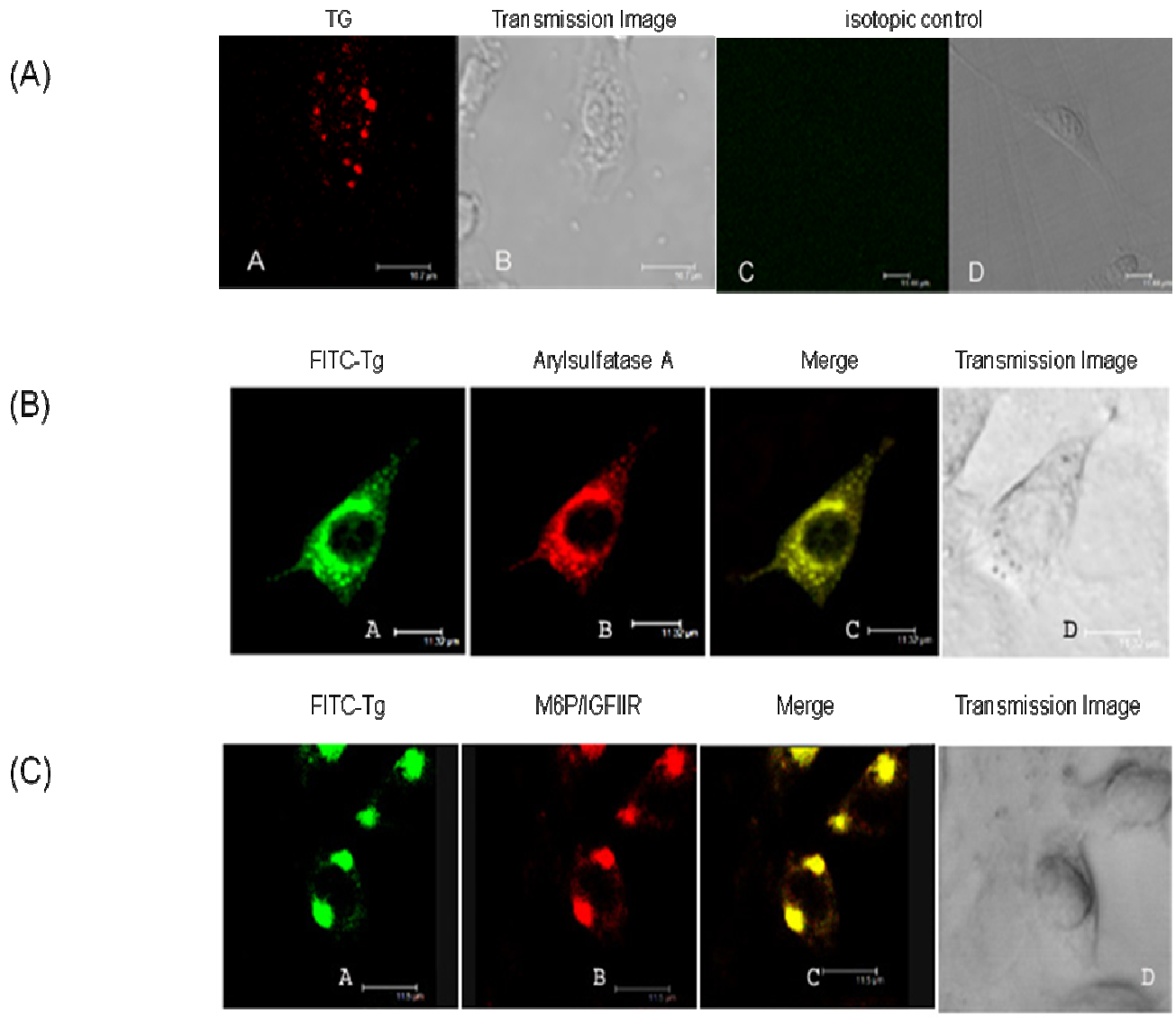

(A)
TG
Transmission Image
isotopic control
A
B
C
D
(B)
FITC-Tg
Arylsulfatase A
Merge
Transmission Image
A
B
C
D
(C)
FITC-Tg
M6P/IGFIIR
Merge
Transmission Image
A
B
C
D

Figure 2.6

SECTION-D

DISCUSSION

The mammalian M6P/IGF-IIR has been characterized as one of the type-1 transmembrane glycoprotein belonging to P-type lectins that is present on the cell surface as well as intracellularly. The bulk of the receptor is localized intracellularly where it binds the newly synthesized lysosomal enzymes via their M6P containing N-linked oligosaccharides for subsequent sorting to endosomes and lysosomes. On the other hand the receptor at the plasma membrane endocytoses secreted lysosomal enzymes. The receptor thus recycles between the *trans-golgi*, plasma membrane and the endosomal compartments. The mammalian receptor has also been characterized as a multifunctional protein, binding to diverse array of ligands (Dahms and Hancock, 2002; Brunett et al., 1994; Gabel et al., 1989; Uta Gasanov et al., 2006). Although the physiological significance of the binding to some ligands is still unclear. The physiological importance of multiple ligands binding to MPR 300 is evident from the fact that MPR 300-deficient mice are not viable. Lethality is apparently due to an impaired regulation of the response to IGF-II, as viability of the MPR 300 knock-out mice is rescued by a simultaneous knock-out of IGF-II.

The mammalian MPR 300 protein was shown to play a role in the clearance and activation of hormones and growth factors, and is able to bind and internalize IGF-II for its delivery to lysosomes in contrast to the second P-type lectin, MPR 46 which is involved in sorting of lysosomal enzymes only. The cDNA clones and partial sequence analysis of the putative receptors have been described in the chicken (Matzner et al., 1996) and the levels of the receptor proteins in different tissues of mammals and chicken vary (Suresh et al., 2006). Further structural analysis of the fish, chicken and the mammalian receptors revealed that the M6P/IGF-II receptor

protein exhibits a conserved cassette structure in the amino terminal region throughout the vertebrates (Udaya Lakshmi et al., 2000). The focus of research in our laboratory is to understand the structure and function of the putative MPR proteins in the animal kingdom. Although it has been well established that the mammalian MPR 300 protein is a multifunctional protein and capable of binding IGF-II and other ligands, it was not clear until recently whether this property is conserved in non-mammalian vertebrates. In a recent study we have shown that purified goat and CEF cell MPR 300 protein are able to bind IGF-II suggesting the possible multifunctional nature of these receptors (Suresh et al., 2006). Since we characterized that the CEF cell MPR 300 protein binding to IGF-II, we set out to analyse whether the multifunctional nature of the MPR 300 protein is also conserved in evolution using the bovine Tg that has been well characterized (Allen et al., 1993; Eduardo et al., 1987) and with the aim to find the possible binding receptor for thyroglobulin on CEF cells. To compare our results with the literature information we used bovine Tg in our studies. In the present study, the first evidence that the purified chicken M6P/IGF-II protein (purified from CEF cells and chicken liver) binds to bovine-Tg in a M6P dependent manner was obtained by passing the radiolabeled receptors on Tg-Sepharose gel. The specificity of the binding has been further substantiated by its elution from the gel by mannose 6-phosphate but not by glucose 6-phosphate. These data suggest that the receptors from both CEF cells as well as from the chicken liver can bind on Tg-gel suggesting Tg as an additional ligand to this receptor protein in CEF cells. [Since we have been using the goat receptors as a mammalian model for our studies, we have also checked if the purified goat MPR 300 protein can bind Tg, and we found that this mammalian receptor also binds Tg, like the human receptor in a M6P dependent manner, [data not shown].

Thyroglobulin binds M6P/IGF-II protein with high affinity and reaches saturation at 66 nM with a K_d value of 33 nM. Preincubation of CEF cells with 5 mM mannose 6-phosphate, anti-goat MPR 300 antibody showed about 80% inhibition where as glucose 6-phosphate, human IGF-II did not show any remarkable inhibition on Tg binding and internalization. In a competitive binding assay with unlabeled Tg there is decline in surface bound radiolabeled Tg showing the competition between labeled and unlabeled Tg to bind M6P/IGFIIR (data not shown). Prior treatment of Tg with Endo H, PNGase F and alkaline phosphatase showed about 90% inhibition. In far-Western blot analysis deglycosylated, dephosphorylated Tg did not show binding with M6P/IGFIIR compared to the untreated Tg. These data suggest that glycosylation and phosphorylation of Tg is important for binding to M6P/IGF-II protein.

Immunofluorescence microscopy studies revealed that the Tg can bind to the M6P/IGF-II protein on the CEF cell surface as detected using a monoclonal antibody to the Tg. Furthermore, experiments with fluorescent labeled Tg in fluorescence microscopy also reveals the clear distribution and colocalization of the Tg with the M6P/IGF-II protein in the cytoplasm and perinuclear region. the detection of arylsulfatase A enzyme in CEF cells in endocytic compartments suggest that these vesicles are late endosomes or prelysosomes because lysosomal enzymes distribution was consistent with the fact that the late endosome and prelysosomal network is the compartment where newly synthesized acid hydrolases containing the M6P marker are sorted from proteins that will be secreted. It is well established in mammals that the M6P/IGF-IIprotein is localized in living cells to different endocytic compartments and the detection of the arylsulfatase A enzyme in these endocytic compartments along with FITC-Tg strongly supports the role of CEF M6P/IGF-II

protein in internalization of this specific ligand. Our experimental data shown in Figure 6, clearly support the idea that Tg taken up by the CEF cells via the receptor is routed to the lysosomes through the intracellular compartments of the endocytic pathway and transported from the early and late endosomes to the prelysosomal vesicles like the arylsulfatase A.

It has been shown that in mammals, in cultured hepatocytes, most of the glycoproteins particularly Tg is internalized either by galactose or asialo-glycoprotein receptors (Ashwell et al., 1982). However such receptors are absent in the chicken liver (Regoeczi et al., 1986; Lunney et al., 1976). Owing to this, it may be possible that the M6P/IGF-II protein might be a candidate for binding and clearance of circulating glycoproteins in the chicken liver. However, detailed and extensive experimentation has to be carried out in order to support this.

Our studies clearly demonstrate that the CEF cells endocytose Tg through the M6P/IGF-II receptor present on the cell surface which is then targeted to the lysosomes for degradation. However it remains to be established whether the results found with the CEF cells, can be extrapolated to the chicken liver hepatocytes. Additionally, it is also not known whether the receptor is present in the thyroidal cells and to what extent? Further studies are necessary to gain new information on these which might give the necessary inputs to study this receptor and its interaction with Tg in this model organism. In summary, this is the first report to provide evidence that the CEF MPR 300 protein can also bind Tg in addition to M6P containing hydrolases and IGF-II. The study thus provides new information on the multifunctional nature of the chicken MPR 300 protein and provides a strong functional significance for the conserved cassette structure of the MPR 300 protein in the vertebrates. Other researchers have already shown that the fish MPR 300

protein also binds IGF-II (Mendez et al., 2001). However it remains to be established whether the fish receptor like the CEF cell receptor can also bind Tg. Table 2.1 describes the comparison of binding abilities of M6P/IGF-II protein from mammalian and non-mammalian species with a number of ligands. Expression of the fugu receptor gene in MPR deficient cells, and analysis of its multifunctional nature such as Tg and other ligands binding, would further provide evidence on the phylogenetic conservation of the multifunctional nature of the MPR 300 protein throughout vertebrates, which is the future direction of work in our laboratory.

Table: 2.1 comparison of ligand binding ability of M6P/IGF-II protein from mammalian and non-mammalian species

M6P-containing ligands	M6P/IGF-II receptor (MPR 300)	
	Mammalian species	**Non-mammalian species (chicken)**
Lysosomal enzymes	Yes	Yes
Transforming growth factor-β precursor (TGF-β)	Yes	ND
Leukemia inhibitory factor	Yes	ND
Proliferin	Yes	ND
Thyroglobulin	Yes	Yes (present study)
Renin precursor	Yes	ND
Dnase I	Yes	ND
CD26	Yes	ND
Epidermal growth factor receptor	Yes	ND
NON M6P-CONTANING LIGANDS		
Insulin-like growth factor II (IGF-II)	Yes	Yes / **R**
Retinoic acid	Yes	ND
Urokinase-type plasminogen activator receptor (uPAR)	Yes	Yes
Plasminogen	Yes	Yes

R- Recent study in our laboratory revealed that the reptilian MPR 300 protein binds human IGF-II and has the critical isoleucine residue as in mammalian protein in the 11th domain.

The following manuscript describing the results in this section has been accepted for publication.

Sivaramakrishna Yadavalli and Siva Kumar Nadimpalli [#] (2008) Characterization of the multifunctional nature of the chicken M6P/IGF-IIR (Mr 300 kDa) protein. ***Proceedings of the A.P.Akademi of Sciences, Journal*.**

Introduction

Preliminary information about the M6P/IGF-II receptor (referred as **CI-MPR** in this chapter) has already been given in introduction of this chapter. The extra cytoplasmic region of the MPR 300 contains 15 repetitive domains that are homologous to each other and to the single domain structure of MPR 46. Domain 3 and 9 bind mannose 6-phosphate while domain 11 has a high affinity binding site for human IGF-II, and is responsible for the internalization and clearance of IGF-II with the help of domain 13 (Devi et al., 1999). Growth factors such as the IGF-I and II have been conserved throughout the vertebrates (Ralphs et al., 1990; Reinecke et al., 1995; Radecki et al., 1997) and play an essential role in biological processes, such as growth, development, and metabolism. IGF-II is a polypeptide mitogen related to insulin that is believed to be particularly important during placental and embryonic development. The CI-MPR mediates endocytosis of insulin-like growth factor II, resulting in growth factor degradation in lysosomes (Nissley et al., 1993; Stewart and Rotwein, 1996). This degradation is an important regulator of growth factor activity *in vivo*, as shown by the phenotype of receptor deficient mice (Lau et al., 1994). A critical isoleucine residue located at position 1572 of the 11^{th} domain of several mammalian CI-MPR proteins has been suggested to be important for IGF-II binding. However in an earlier study it was shown that purified CIMPR protein from chicken liver and *xenopus*, showed weak binding to IGF-II (Clairmont and Czech, 1989, Canfield and Kornfeld, 1989). Subsequent cloning and characterization of the chicken CI-MPR revealed that the mannose 6-phosphate binding regions of the receptor are same as in the mammals, but in the IGF-II binding domain the isoleucine located at 1572 position is replaced by leucine in chicken and the weak binding of the chicken protein to IGF-II has been attributed to this change (Zhou et

al., 1995). Therefore it was proposed that the acquisition of an IGF-II binding site by the CI-MPR is a late event in evolution. In a separate study it was shown by Mendez et al., (2001) that the fish CI-MPR can bind IGF-II with high affinity. Other parts of the IGF-II binding domain may also play an important role in binding IGF-II and the IGF-II binding property appears to have occurred after the divergence of marsupial and placental mammals from their common ancestor with egg-laying mammals, and it has been suggested that this acquisition was a major factor in driving the evolution of an imprinted CI-MPR in some mammals. After the work of Mendez et al, (2001) we have also found that the chicken CI-MPR which falls above the fish in the evolutionary tree also shows IGF-II binding affinity (Suresh et al., 2006).

Towards understanding the evolution of the MPRs, research work in our laboratory has been focused on the identification, purification, biochemical characterization, cloning and sequencing of the receptors from non-mammalian vertebrates. Although the reptilian MPRs have been purified from *calotes* liver tissue and shown to be homologous to the mammalian proteins (goat) there have been so far no detailed studies on the structural and functional characterization of the reptilian receptors. Mammalian homologues of the receptors were identified by us from amphibians, fish and also in the invertebrate molluscs. Extensive characterization of the early vertebrate zebra fish MPR 46 revealed that the receptor is conserved throughout the vertebrates (fish to mammals). Studies carried out by other workers on Zebra fish MPRs also revealed that the MPR 300 also exhibits conserved structural domains (Catherine et al., 2006). These studies point towards the conserved m6p binding domains (3 and 9) from fish to mammals. However no such conclusive evidence has been shown so far for the IGF-II binding domains among the different vertebrate CI-MPRs. Therefore, it is important to gain new insights into the IGF-II binding domain

of the vertebrate CI MPR's to establish the evolution of the receptor and functional significance of its domain structures. Therefore the present study was carried out with the following objectives. i) to localize the CIMPR protein in the *calotes* liver tissue, ii) to analyze the interaction between the human IGF-II and purified reptilian *calotes* CIMPR protein and iii) to clone the 11^{th} domain of the *calotes* MPR 300 using an RT-PCR approach and make a structural comparison of the sequences of the available IGF-II domains in the vertebrates.

MATERIALS

Garden lizards liver was collected from a local animal supplier. From some animals, the testes were separated. Both the liver and testes were kept frozen at minus 80 °C, Affinity purified antibody to the goat MPR 300 protein was obtained as described earlier (Suresh et al., 2002).

METHODS

Tissue localization of the CI-MPR

Calotes were anaesthetized with chloroform and received a retrograde perfusion through the abdominal aorta with phosphate buffered saline for 1 min immediately followed by 4% Para formaldehyde in 0.1 M phosphate buffer at pH 7.4. After 5 min of perfusion, portions of two liver lobes were excised and were cut into 1 mm thin slices that were immersion-fixed in the same fixative for an additional hour. For immunofluorescence semithin (20 µm) cryo sections from prefixed livers were prepared on microtone. For double immunofluorescence staining, liver sections (20 µm) were incubated overnight with a combination of anti-CI-MPR antibodies (1:300) and anti-LAMP1 (1:200), After incubation with the primary antiserum, sections were rinsed with PBS, exposed to FITC- or TRITC-conjugated secondary antibodies (1:200) for 1h at room temperature, washed thoroughly with PBS and then cover-slipped with Vectashield mounting medium. Immunoastained sections were examined under a Leica fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera.

Quantification of CI-MPR in different tissues of garden lizard

Quantification of the receptor in membrane extracts of different tissues was done by ELISA as described in (Suresh et al., 2002).

Purification of CI-MPR on PM-affinity chromatography

Membrane proteins extraction, purification of the receptor on Sepharose-Divinyl sulfone-phosphomannan affinity matrix, analysis of the purity on SDS-PAGE and western blot was done as described in (Siva Kumar et al., 1997)

Protein estimation

Protein estimation was done using bicinchoninic acid reagent following manufacturer's instructions (Sigma, USA).

Ligand blot assay

Ligand blot analysis between CI-MPR 300 and biotinylated IGF-II was done as described (Mendez et al., 2001)

Affinity cross-linking of biotinylated IGF-II with purified reptilian CI-MPR

Cross-linking studies were carried out as described (Causin et al., 1989). About 5 μg of purified receptors were acetone precipitated and the precipitate was solubilized in 37μl of 50 mM-sodium phosphate, pH 7.4, containing 150 mM NaCl, 0.1% Triton X-100 and 0.1% bovine serum albumin and incubated for 20 min at 4^{o}C with or without 40 nM unlabelled IGF-II followed by 10μl of biotinylated IGF-II, (40 nM) was added and incubated for 3h at 4^{o}C. Cross-linking of bound IGF-II with receptor was performed with 1mM-DSS by incubating the reaction mixture for 15 min at 4^{o}C. The reaction was stopped with 5 μl of 1 M-Tris/HCl pH 7.4, 2.75 μl of 20% SDS and 3 μl of glycerol (100%). The reaction mixture was heated at 95^{o}C for 5 min. and the samples were subjected to 7.5% SDS-PAGE. The bands were visualized by ECL method using a Kodak X-ray film with light intensifying screens.

ELISA binding assay to determine dissociation constants (Kd) for the binding of CI-MPR to IGF-II

A novel ELISA based immunoassay (Heuer et al., 1996) was used to measure the binding affinity of CI-MPR protein to HIGF-II. ELISA assays were performed with purified CI-MPR directly in Corning 96-well polystyrene disposable sterile ELISA plates. Purified CI-MPR (100 ng), in 100 µl of interaction buffer (20 mM Tris (pH 8.3), 150 mM NaCl, 0.2% Tween 20), was adsorbed to the wells of an ELISA plate by overnight incubation at 4 °C. Subsequently, the wells were washed thrice with interaction buffer. The wells were then blocked by incubation with the blocking buffer (interaction buffer containing 5% bovine serum albumin) for 1h. Then the blocking buffer was removed, and the wells were added with 5-Fold serially diluted biotinylated HIGF-II protein in 100 µl of interaction buffer plus 0.1% bovine serum albumin and proteins were allowed to interact with plate-bound purified reptilian CI-MPR for 1 h at room temperature. Following the interaction period, unbound IGF-II protein was removed, and the wells were washed with interaction buffer. Following this, streptavidin coupled with horseradish peroxidase (HRP) (Bangalore genie), was applied to the wells for 1 h, and unbound HRP-conjugated streptavidin was removed, by washing the wells with wash (PBS with 0.1% BSA) buffer, and the receptor-ligand complex in the wells was detected using the chromogenic substrate TMB/H_20_2 ,colour development was stopped after 15 min by adding 1 N HCl, and the absorbance was measured at 450 nm using a Bio-Rad model 450 micro plate reader.

Reverse Transcription-Polymerase Chain Reaction and Sequence Analysis

The cDNA sequences for the putative IGF-II binding region was obtained using Total RNA extracted from garden lizard liver, followed by reverse transcription-polymerase

chain reaction (RT-PCR). Total calotes liver RNA was extracted using the RNeasy Mini Kit, and first-strand cDNA was synthesized using 0.5 ng of oligo (dT) primer and 200 units of Reverse transcriptase enzyme in a total volume of 20 µl (according to the manufacturer's protocols).

Primer design and amplification

Amplification of the putative IGF-II binding region was performed using two non-degenerate oligonucleotide primers that were based on the conserved cDNA sequence of the other known CI-MPR. The primer pair 5'-ATCAATGTCTGCAA-3' (IGF-1) and 5'CGTCCAGGAGAA-3' (IGF-2) amplify a 474-base pair fragment. The PCR was carried out in a total volume of 20 µl, 1µl (10pM) of primers, 2 µl of oligonucleotides, 4.5 µl of cDNA template,11.5 µl nuclease free water and 1 µl of Taq DNA polymerase. After Taq DNA polymerase was activated by heating at 95°C for 5 min, the PCR reaction proceeded for 38 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing), and 1 min at 72 °C (extension). After electrophoresis and ethidium bromide staining, the correct sized (474bp) fragment was excised from the agarose gel, purified using Qiagen PCR DNA Purification System, and cloned into the T/A cloning vector (pTZ57R).

Cloning and transformation of cDNA of 11th domain

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

DNA sequencing

DNA sequencing was done at Biosereve sequencing Pvt.Ltd.sequence comparisons were performed with the CLUSTAL W method available online at http://www.justbio.com.

RESULTS

Purification

The CI-MPR from the *calotes* liver membrane extracts was affinity purified using phosphomannan-Sepharose gel. The bound protein was eluted from the gel with 5 mM mannose 6-phosphate, concentrated by acetone precipitation. The protein concentrations were analyzed using BCA reagent. In SDS-PAGE a single band at 300 kDa was observed corresponding to the receptor. The purified receptor was used for further experiments.

Localization of MPR 300 in liver tissues of calotes

In our previous studies (Siva Kumar et al., 1997), we have confirmed that an antiserum to the purified goat CIMPR recognizes specifically the receptor from *calotes*. Affinity purified goat MPR 300 antibodies have been used in immunochemical staining of the liver sections in this study. A western blot with the purified receptors was also done to re-confirm their cross-reactivity (data not shown). Additionally we performed the localization of CI-MPR in liver tissues by immunofluorescence, where the semi thin cryo section of garden lizard liver tissue was processed as described in methods and probed with goat CI-MPR antibody along with a lysosomal marker protein LAMP1 and we were able to detect the fluorescence along the entire plasma membrane in addition to the pre-lysosomal compartments. In the peripheral cytoplasm flocculent fluorescence occurred, which relates to MPR reactivity as is also evidenced by the perfect co-localization with LAMP1 protein. Furthermore from (Fig.2.7A) it is apparent that the MPR label was also distinctly seen in the early endosomes as observed by immunofluorescence.

Quantification of CI-MPR

In order to quantify the receptor that could be detectable with the goat MPR 300 antibodies, an ELISA method was developed. As shown in Fig. 2.7B, it is clear that the liver tissues has high amount of MPR 300 ~ 1.7 ng per µg of membrane proteins followed by other tissues like brain, heart, ovaries, testis spleen and kidney. The level of detection of MPR 300 was done using a standard graph range in between 1-10 ng.

Ligand blot assay

Specific interaction of the receptor to IGFII was analyzed in a ligand blot assay. The CI-MPR was separated on a 7.5 % SDS-PAGE and transferred to PVDF membrane. The membrane was probed with biotinylated human IGF-II followed by incubation with strepatavidin conjugated HRP, washed and developed with ECL reagent. Protein bands were visualized at 300 kDa. From Fig 2.8A it is apparent that the band represents purified goat (control, lane 1) and garden lizard MPR 300 showed binding to IGF-II (lane 2). This suggests that there is an interaction between the receptor and the HIGF-II. The specificity of the interaction was evidenced by the competitive displacement seen by the excess addition of unconjugated IGF-II (Fig 2.8B, lane 2), and the inability of 5 mM mannose 6-phosphate or insulin (400nM) to completely displace biotinylated IGF-II (Fig 2.8B lanes 1 and 3).

Cross-linking experiments

In a cross-linking experiment, the purified reptilian CI-MPR and biotinylated IGFII with the cross-linker DSS, gave a protein band of 307 kDa (Fig. 2.8C, lane 1), while in absence of cross-linker DSS, receptor alone exhibited a molecular mass of 300 kDa (Fig. 2.8C lane 2). Both bands could be detected using affinity purified goat MPR 300 antibody that can recognize the reptilian receptor.

ELISA binding assay to determine dissociation constants (Kd)

To determine the overall binding affinity of CI-MPR and IGF-II, an ELISA-based immunoassay (Heuer et al., 1996) was used, where purified receptor was coated onto the wells of ELISA plates and allowed to interact with different concentrations of biotinylated human IGF-II (5-60nM). After extensive washing, the amount of IGF-II bound to CI-MPR was assessed using IGF-II specific antibodies which in turn, were reacted with HRP-coupled secondary antibody or directly by HRP-coupled secondary antibody. Bound HRP was detected and quantitated using the chromogenic substrate TMB (where colour development was directly proportional to IGF-II bound to M6P/IGFIIR 300 (see "Experimental Procedures") and was used to generate the binding curve (Fig 2.9 inset). Kd values for IGF-II and binding to CI-MPR were derived by Scatchard plot analysis of the binding curves (Fig 2.9). The slope of the Scatchard plots indicated that binding of IGF-II to CIMPR follows monophasic kinetics. The calculated Kd value for IGF-II binding to CI-MPR was 12.02 nM (moderate affinity compare to mammals)

RT-PCR and Sequence Analysis

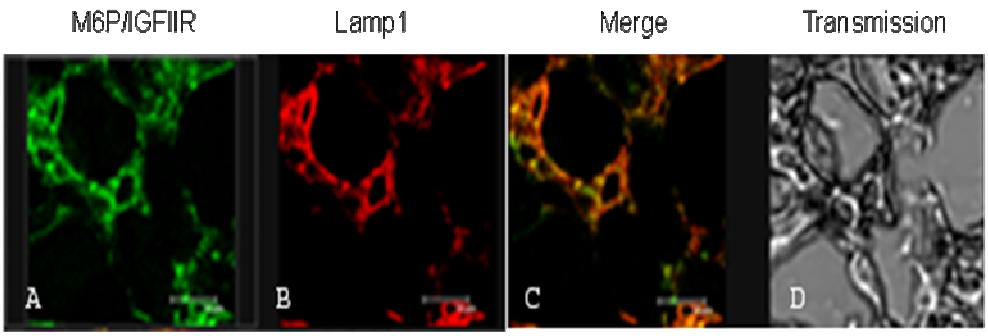
Total RNA isolated from *calotes* liver was amplified using an RT-PCR approach as described under methods, to give a 474 bp fragement (Fig.2.10B) that was isolated, cloned and sequenced (Fig.2.10C). the deduced amino acid sequence of the proposed IGF-II binding domain residues 1508–1665 (Fig.2.11), revealed that there is a 55-60% identity between the reptilian and mammalian CI-MPRs (Human, bovine, rat and mouse) and a 45-50% identity with monotremes and egg-laying mammals, 38% identity with chicken receptors and 30-35% identity between *calotes* and fishes (Fig.2.12). Interestingly, multiple sequence alignment of the of the reptilian 11^{th} domain with other known CI-MPR 11^{th} domains reveals that the amino acid,

corresponding to Ile^{1572} of the human CI-MPR, which has been implicated for direct involvement in ligand binding, is also present in *calotes* (Fig.2.12). Recent study by (Oliver et al., 2006) indicates that there are other regions in 11^{th} domain of the mammalian CI-MPR that also contribute to the binding of the IGF-II. From our results it is seen that the in *calotes* these regions are also conserved.

Figure 2.7: (A) Localization of CI-MPR in reptilian liver sections. Semi-thin (200 nm) cryosection of prefixed liver cells were immunolabeled with anti-polyclonal Goat MPR 300 antibody (A) and polyclonal LAMP1 antibody (B) followed by FITC and TRITC labelled secondary antibodies respectively. Colocalization of the receptor and LAMP1 (yellow colour in C merge of A and B.) was seen within early or pre-lysosomal compartments. (*Bars 10.5µm*).

(**B).** Quantification of CI-MPR levels in different Tissues of reptilian (*Calotes*) – Experimental details given under methods.

(A)


M6P/IGFIIR
Lamp1
Merge
Transmission
A
B
C
D

(B)

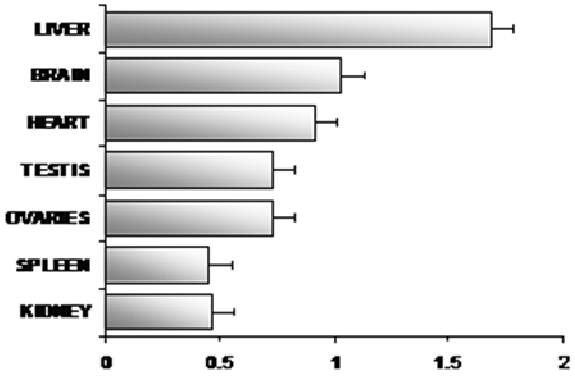

LIVER
BRAIN
HEART
TESTIS
OVARIES
SPLEEN
KIDNEY
0
0.5
1
1.5
2

Figure: 2.7

Figure 2.8: Ligand blotting-(A). Ligand blotting of reptilian CI-MPR with HIGF-II. Purified receptor after separation by SDS-PAGE was transferred to a PVDF membrane and incubated with 40ng of biotinylated HIGF-II, followed by incubation with streptavidin-HRP conjugated secondary antibody and developed using ECL reagents. Lane 1, goat protein (control), lane 2, reptilian protein.

B). Ligand blotting in presence of 5mM mannose 6-phosphate (lane 1) (faint bands below represents possible proteolytic products of the receptor). Non-biotinylated HIGF-II (lane 2) and Insulin (400nM) (lane 3).

C). Cross-linking of reptilian receptor with HIGF-II. After cross-linking, samples were separated on a 7.5% SDS-PAGE, transferred onto PVDF membrane and blot was incubated with affinity purified goat MPR 300 antibody and developed with ECL. Hollow arrow (lane 1) indicates the position of cross-linked product and solid arrow indicates reptilian MPR 300 (uncross-linked).

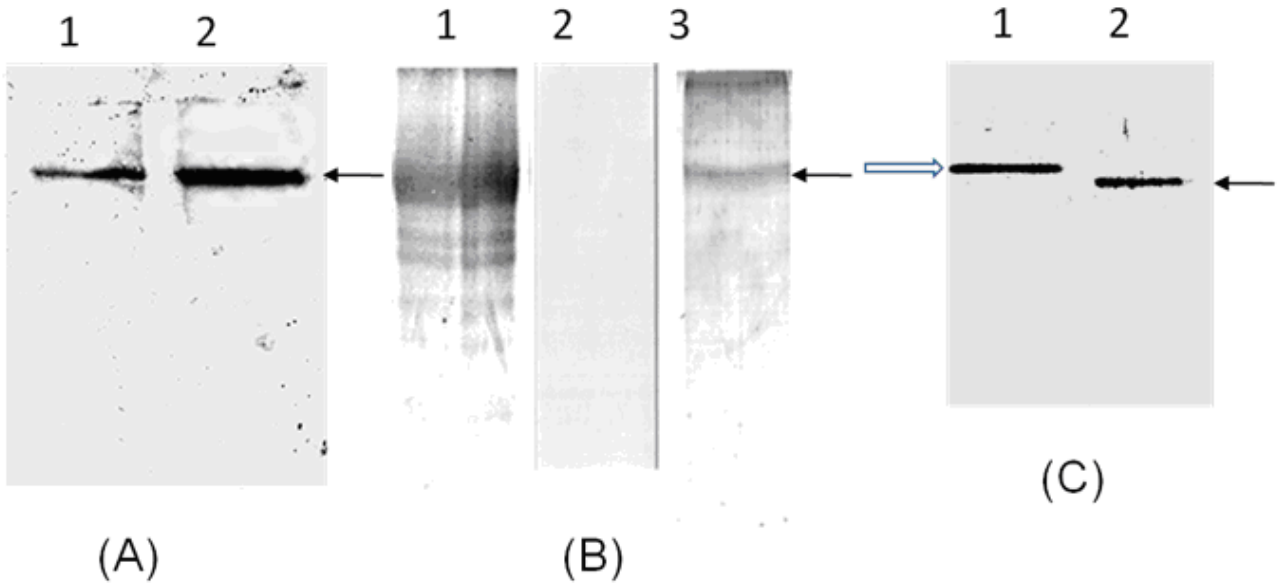


Figure 2.8

Figure 2.9: Determination of dissociation constants (Kd) for CIMPR interaction with HIGF-II. Binding curves were obtained for CIMPR interaction with HIGF-II using the ELISA binding assay as described under “Experimental Procedures.” Each data point on the curve represents the least mean square values obtained from three independent experiments done in duplicate. The CIMPR and HIGF-II binding curves (inset) were obtained using 100 ng of membrane proteins of reptilian Liver coated onto the wells of the ELISA plates. Binding curve was used for Scatchard analysis, where the slope was used to calculate the inverse of the dissociation constant (Kd) values.

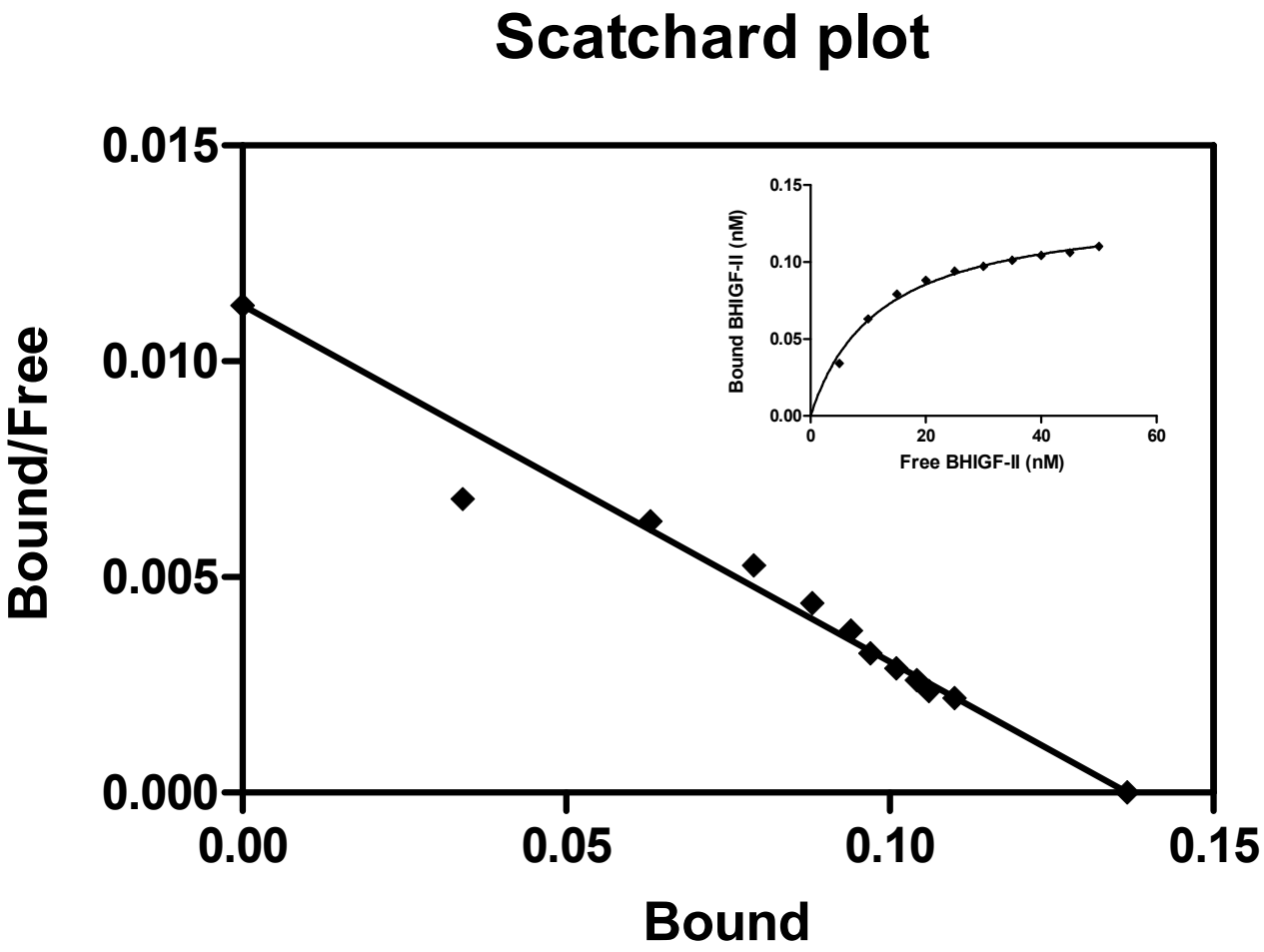

Scatchard plot
Bound/Free
0.015
0.010
0.005
0.000
0.00
0.05
0.10
0.15
Bound
Bound BHIGF-II (nM)
0.15
0.10
0.05
0.00
0
20
40
60
Free BHIGF-II (nM)

Figure: 2.9

Figure 2.10: Molecular cloning of 11th domain of reptilian liver CI-MPR (details given in methods). Schematic representation of molecular cloning of the reptilian CI-MPR 11th domain into pTZ57R.

A). 1% Agarose gel electrophoresis of PCR amplified fragments Lane 1, standard DNA ladder (100 to 10,000 bp), Lane 2, and PCR amplified fragment (474bp)

B). Restriction digestion analysis. Lane 1 DNA ladder, Lane 2 is digested vector (fragment released from the vector after digestion with EcoRI and HindIII), Lane 3 is single digested vector and Lane 4 is undigested vector.

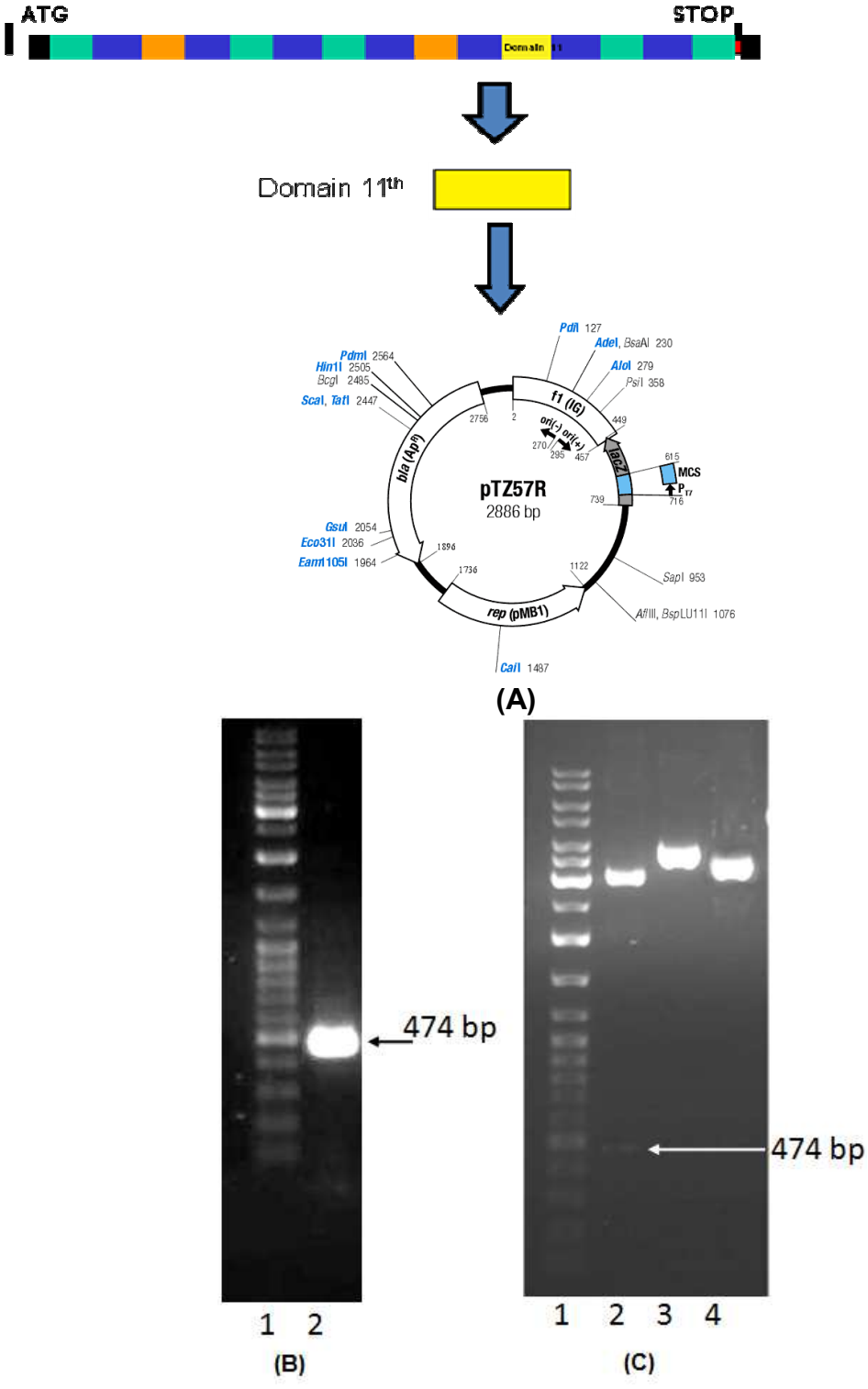

ATG
STOP
Domain
Domain 11th
pTZ57R
2886 bp
bla (Ap^R)
f1 (IG)
rep (pMB1)
lacZ
MCS
PdiI 127
AdeI, BsaAI 230
AloI 279
PsiI 358
PdmI 2564
Hin1I 2505
BcgI 2485
ScaI, TaiI 2447
GsuI 2054
Eco31I 2036
Eam105I 1964
SapI 953
AflIII, BspLU11I 1076
CaiI 1487
(A)
474 bp
1 2
(B)
474 bp
1 2 3 4
(C)

Figure: 2.10

Figure 2.11: Nucleotide and deduced amino acid sequence of the 11th domain of reptilian CI-MPR.

```
  1 - AAGAGCAACGAGCATGATTATGATGTCTTCAGCAACCCAAGCACAGGACACCTGGACGTA - 60
  1 - K  S  N  E  H  D  Y  D  V  F  S  N  P  S  T  G  H  L  D  V   - 20

 61 - CTGGAAACAGTACGATTTAGGGCGGGATTCATAATGGCTTACTCCGAGAAGGGGTTGGTT - 120
 21 - L  E  T  V  R  F  R  A  G  F  I  M  A  Y  S  E  K  G  L  V   - 40

121 - TTCATGACGCGACTTGGGGCCGCTCATCCGTGCCCTCATCACGTGGGGGCCTGCTTTGGA - 180
 41 - F  M  T  R  L  G  A  A  H  P  C  P  H  H  V  G  A  C  F  G   - 60

181 - GACACAAGGATCAACGTGGGCCCAGCCAATAAGAGATTCGGGTACGTGGACCAGGTCCAT - 240
 61 - D  T  R  I  N  V  G  P  A  N  K  R  F  G  Y  V  D  Q  V  H   - 80

241 - GATCTGGTGTACAAGGATGGGTCCCCTTGTCCCTATAAAAGTGGCCTGAGCTGCAAGAAC - 300
 81 - D  L  V  Y  K  D  G  S  P  C  P  Y  K  S  G  L  S  C  K  N   - 100

301 - GTGATCAGTGAGGTGAACAGGACTGAGGCCATACCAAGGAATCGGCCCTACCTCACTTCC - 360
101 - V  I  S  E  V  N  R  T  E  A  I  P  R  N  R  P  Y  L  T  S   - 120

361 - CGTGACCTCCAGGTCTGCACCCTCACTTTCGGGTGGGATACGGGGCTGGGTTGCGAGCAA - 420
121 - R  D  L  Q  V  C  T  L  T  F  G  W  D  T  G  L  G  C  E  Q   - 140

421 - GCGACCTTATTCGTCACGAGAAATGTAAGTTCCATTGTTGACTTGTCTCCCCTT - 474
141 - A  T  L  F  V  T  R  N  V  S  S  I  V  D  L  S  P  L       - 160
```

Figure: 2.11

Figure 2.12: Multiple sequence alignment of amino acid sequences of the 11th domain of CIMPR from different animal species. ($) indicates the conserved IGF-II ligand binding residues ;(*) marks identical amino acid residues;(:) related amino acid residues;(.) Predominantly the same amino acid residue; residues in red colour indicates **AB loop**; residues in blue colour indicates **CD loop** and residues in green underlined indicates **FG loop**.

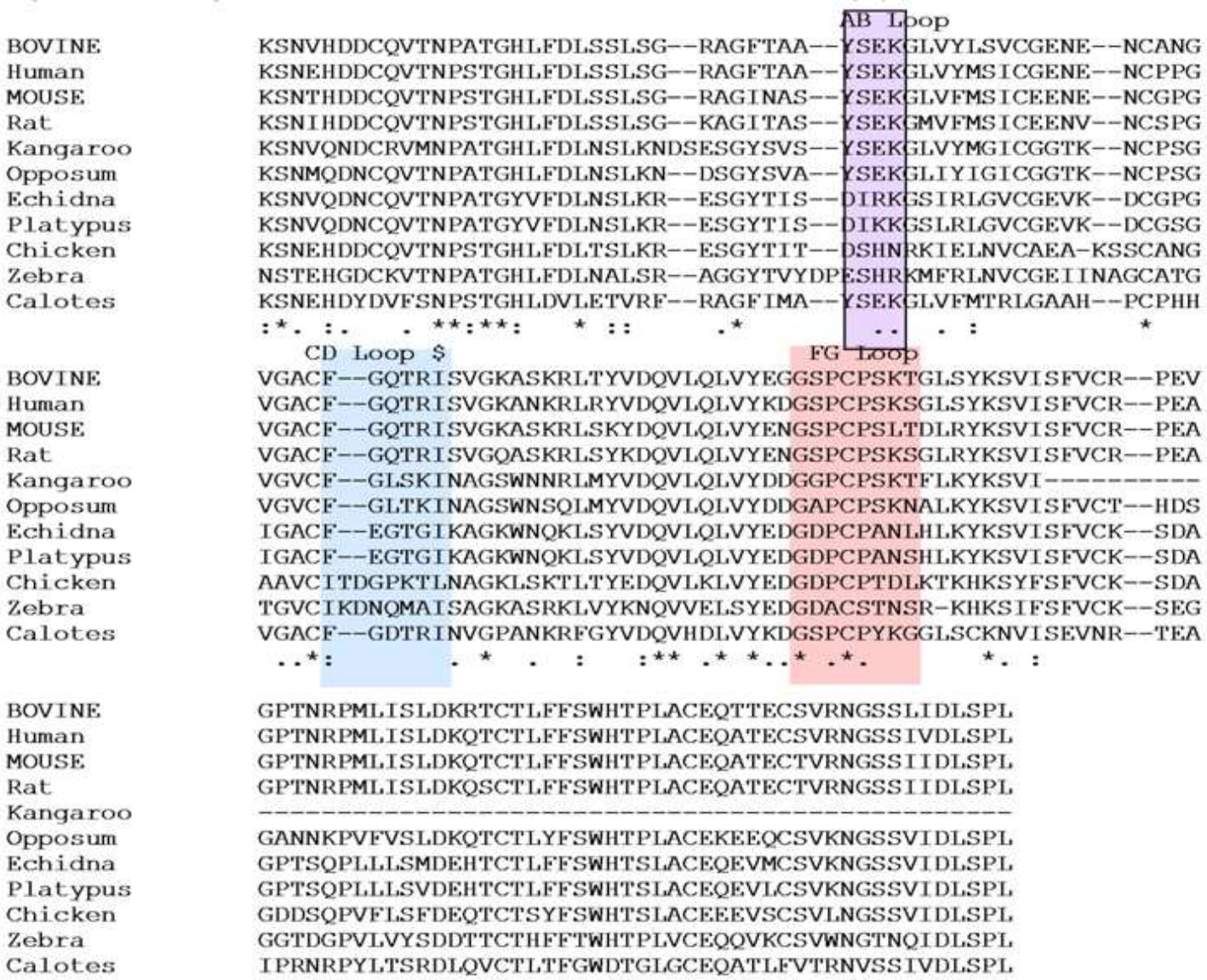

AB Loop
CD Loop $
FG Loop
BOVINE
Human
MOUSE
Rat
Kangaroo
Opposum
Echidna
Platypus
Chicken
Zebra
Calotes

Figure: 2.12

DISCUSSION

Lysosomes in eukaryotes play an important role in the degradation of biological materials by hosting a large number of hydrolytic enzymes. A number of lysosomal diseases have been identified in humans and the story of lysosomal biogenesis has become highly complex in the recent past with identification of several novel proteins involved in the mammalian lysosomal biogenesis. The lysosomal enzymes shortly after their synthesis and processing in the *trans*-Golgi Network encounter two homologous proteins that exhibit distinct functions. These proteins designated as the CI-MPR and CD-MPR proteins are known to play a crucial role in targeting of lysosomal enzymes to their destinations. Compared to the depth of information available on the mammalian MPRs and their role in lysosomal biogenesis, the role of the receptors in the non-mammalian vertebrates is limited. The main focus of research in our laboratory is to understand the structure and function of the receptor proteins in the non-mammalian vertebrates and invertebrates to finally understand and establish the evolution of these interesting proteins. We made several first contributions to identify the mammalian homologues of the putative receptors among a number of non-mammalian vertebrates (fish to mammals, animal species and cell lines) and from the invertebrates (echinodermates and molluscs) and genome sequences of several model organisms have been elucidated in the past few years and many more are being sequenced now. The sequences available have provided several avenues to clone, express and characterize biologically important proteins and to understand their structure-function relationships. A recent classical example pertaining to the Mannose 6-phosphate receptors is on the functional characterization of the zebra fish receptors elucidating their role in the transport of lysosomal enzymes (Suresh et al., 2006; Catherine et al., 2006). These studies

suggest evolutionary conservation of the vertebrate (fish to mammals) mannose 6-phosphate binding sites. An important unanswered question to date is whether the IGF-II binding domain of the CI-MPR like the m6p binding domain is also conserved throughout the vertebrates (fish to mammals). To address this question and to gain new insights into the evolutionary conservation of the vertebrate receptor IGF-II domain structure, the present detailed study was undertaken. It would be logical to study this using an animal model from the reptiles such as the *calotes*, due to their position in the evolutionary tree and since there are no reptilian cells available to us we chose to carry out our studies using the animal model *calotes* from which we already characterized the CI-MPR. The strategy adopted was to first identify if the purified reptilian CI-MPR can bind human IGF-II by *invitro* analysis and second to make the first cDNA clone for the reptilian IGF-II binding domain of the receptor that would allow us to make a structural comparison of the sequences of the reptilian protein to other known receptors and to understand its evolutionary conservation among the vertebrates.

IGF's (IGF-I and II) in the reptiles and other non-mammalian vertebrates have been characterized as peptides that play a critical role during early development (Ralphs et al., 1990; Reinecke et al., (1995). Though we reported the existence of the CI-MPR protein in reptiles earlier and its ability to bind phosphomannan gel in a mannose 6-phosphate dependent manner [thereby suggesting it to be a lysosomal enzyme sorting receptor like the mammalian protein], here in this study we present the first report that the receptor also binds IGF-II and further extend our studies using cloning strategy to elucidate that the reptilian protein has conserved amino acid sequences in domain 11^{th} like the mammalian protein. Homologous proteins have been identified by us from molluscs and more recently from the echinodermates,

only recently we established the function of the echinodermate MPR 46 protein as a lysosomal enzyme targeting receptor (*YSRK, NSK- submitted revised version to glycoconjugate journal, May 2008*). Below the molluscs in the *Drosophila melanogaster* no MPR 46 could be detected, and only a lysosomal enzyme receptor protein (LERP) was characterized from the *Drosophila.* LERP is a glycoprotein with an apparent molecular mass of 150 KDa and it appears as a truncated form of the mammalian CI-MPR. The repeat 3 of the LERP corresponding to domain 11 of the human CI-MPR exhibits highest homology to the human receptor. However in *Drosophila* so far no genes coding for IGF-II or IGF-I have been identified. We have already established that LERP doesn't bind the multivalent phosphomannan (Dennes et al., 2005), a property exhibited by the CI-MPR from mammals to molluscs and it is unlikely that the LERP would bind human IGF-II although this has to tested. In the other arthropods such as the prawn, and in annelidae (earthworm) we could not detect MPR 46 and identified only MPR 300 like polypeptides (Raju et al.,2001), Thus from, our studies we suggest that the mannose 6-phosphate recognition and lysosomal enzyme sorting by the receptors is conserved from fish to mammals. Among the two receptors only mammalian MPR 300 has been shown to be a multifunctional protein till recently, and binds ligands independent of divalent metal ions. In addition to the mannose 6-phosphate (M6P) containing ligands, it also binds human IGF-II. Additional ligands that the mammalian CI-MPR has been shown to bind are thyroglobulin, retinoic acid, epidermal growth factor, proliferin, rennin precursor, leukemia inhibitory factor, and precursor form of transforming growth factor, Granzymes A and B, DNase I and CD26 (Hawkes and Kar, 2004). Mendez et al., 2001 have shown that the teleost fish embryo CI-MPR binds IGFII, and we also found that goat and chicken CI-MPR bind IGF-II (Suresh et al., 2006).

These results suggest that possibly the multifunctional nature of the receptor is also conserved in the vertebrates. In separate studies it has been found that the zebra fish MPR 300 (Catherine et al., 2006) and the fugu fish MPR 300 (Suresh et al., 2005), exhibit conserved mannose 6-phosphate binding sites throughout the vertebrates. However the IGF-II binding site residue which has been shown to be critical for binding IGF-II in mammals (Ileu 1572) though present in both the fish species is at a position different from that found in mammals. It still remains to be established whether these fish species bind IGF-II. In the present study, first we found that the purified reptilian MPR 300 can bind IGF-II in a cross-linking experiment. Second we could localize the receptor in the semi-cryo liver tissue slices of the *calotes* together with the LAMP 1 protein as visualized by confocal microscopy. Furthermore, the liver tissues express a high amount of M6P/IGFIIR when compared with other tissues like brain, heart, kidney, ovaries, testis and spleen. Additionally, in an ELISA binding assay purified *calotes* MPR 300 protein showed binding to the biotinylated human IGF-II .From these, a Kd value of 12.02 nM for the *calotes* CI-MPR were obtained. The Kd values of human, rat, bovine and opossum were reported to be 1, 1-2, 0.2 and 14.5 nM, respectively (Dahms et al., 1993) and in our previous study, we found the Kd values for goat and chicken receptors to be 12.5 and 11.1 respectively, and for fish it was found to be 0.12 nM (Mendez *et* al., 2001). In recent years, biotinylated ligands have been employed as sensitive reagents to study protein-ligand interactions, and the specific interactions can be visualized after treatment of the product with streptavidin-coupled HRP and enhanced chemiluminiscence reagent. The specific interaction of the IGF-II with the reptilian receptor was also confirmed by the reactivity of the receptor with biotinylated IGF-II. Neither mannose 6-phosphate nor insulin had any effect on this

specific interaction. The efficient binding of the receptor to biotinylated IGF-II in these assays further supports the above experimental findings that the MPR 300 from *calotes* indeed binds human IGF-II. Thus, the results of our experiments with the purified *calotes* receptor are consistent with the published information on the binding of several CI-MPR proteins to IGF-II (Suresh et al., 2006). To further support the data obtained for *calotes* CI-MPR 300, we performed the cDNA cloning and sequencing of 11th domain of *calotes* using total *calotes* liver RNA. Sequence analysis of this suggests that the reptilian species exhibits extensive sequence homologies in the 11th domain as compared to several mammalian species. In particular the isoleucine residue that has been shown to be conserved in several mammals is also conserved in the reptiles. However the extent of homology to chicken and fishes is lower compared to mammals. Recent studies indicate those two hydrophobic residues in the CD loop (F1567 and I1572) and further a non-hydrophobic residue (T1570) that can slow down the disassociation, the receptor and ligand. Additionally, Y1542 and E1544 of AB loop and the two proline residues (P1597, P1599) located in the FGloop (shown in the Figure comparing the sequences of the 11th domain) that have been shown to be involved in IGF-II binding of other species are also conserved in the *calotes.*

We report here the specific binding abilities of CI-MPR with IGF-II in a heterologous system. Endosomes and lysosomes regulate the activity of critical signaling molecules in development and may play a role in and development (Piddini and Vincent, 2003). Several studies point to the fact that in mammals the critical IGF-II levels are essential in the normal development and the CI-MPR is also developmentally regulated to maintain the levels of IGF-II and plays key role in development of the organism in non-mammalians and mammals (Mendez et al.,

2001). From the new data generated in the present study, it is logical to hypothesize that the significance of the interaction of IGF-II with CI-MPR might be an important phenomenon in the development of reptiles as well, a thought that needs further validation.

In summary, this is the first study reporting on the localization of the CI-MPR protein and its quantification in different tissues of the non-mammalian vertebrate *calotes*, and the first cloning, sequencing and functional analysis of the reptilian IGF-II binding domain of CI-MPR paving way to gaining new insights into the IGF-II binding property of the CI-MPR 300 protein in the *calotes*. Extensive comparative studies strongly suggest that in the vertebrates (fish to mammals), the CI-MPR protein exhibits conserved repetitive cassette structures in the extra cytoplasmic domain. While earlier studies established the conserved M6P binding domains of the vertebrate receptor, the present study describes and establishes the importance of the 11^{th} domain of the *calotes* and resolves the important question on the evolutionary conservation of the vertebrate IGF-II domain structure. The study demonstrates that in the vertebrates, the same receptor protein functions in lysosomal enzyme and IGF-II binding suggesting the multifunctional nature and evolutionary conservation of the vertebrate CI-MPR. Further studies are needed to establish the biological significance and the physiological role of IGF-II binding to the CI-MPR in *calotes*. Our present and future work is focused to obtain the sequences of the mollusc receptors (MPR 46 and 300) to find out if the M6P and the IGF-II binding domains are also conserved in respective receptors in these species, which would further establish the evolution of the receptors in the animal kingdom.

A manuscript describing the findings in this section is being communicated to Development, Genes and Evolution.

Sivaramakrishna Yadavalli, Amancha praveen kumar and Siva Kumar Nadimpalli [#] (2008) Vertebrate M6P/IGFIIR : New insights into the evolutionary conservation of the IGF-II binding domain ***Development Genes and Evolution.***

INTRODUCTION

In mammals, mannose 6-phosphate receptors (MPRs) target acid hydrolases to endosomes and lysosomes, but nothing is known of MPRs role in acid hydrolase sorting in non-mammalians. MPRs have been identified in chicken embryonic fibroblast cells, little is known about their cellular localization and their *invivo* functions. Here, we describe the functions of the CEF cell cation-independent MPR (CI-MPR) *in vivo.* To assess the role of MPR 300 in the sorting and trafficking of lysosomal enzymes the function of MPR 300 was abolished by the use of RNAi technology. We created stable CEF cells transfected with the psilencer 1.0 U6 vector containing a selected siRNA template oligonucleotide (small hairpin interference RNA) where the levels of MPR 300 were found to be suppressed. The elimination of MPR 300 by this method will permit to determine whether or not MPR 300 is involved in a general mechanism of lysosomal sorting that involves the trafficking of various soluble lysosomal proteins to lysosomes in CEF cells. In CEF cells the CI-MPR 300 is largely associated with the *trans*-Golgi Network (TGN)/late endosomal compartments. Knockdown of CI-MPR 300 expression using small RNAi molecules results in the secretion of large amounts of newly synthesized lysosomal enzymes into the medium thereby disrupting the catabolic function of lysosomes. The present study revealed that the intracellular levels of lysosomal proteins decreased to <15%, and undigested material accumulated in the lysosomal compartment. Thus, CI-MPR 300 has a role in intracellular sorting of lysosomal enzymes to lysosomes from the TGN in CEF cells and also in the internalization of mannose 6-phosphorylated ligands.

MATERIALS

Affinity purified antibody to the goat MPR 300 protein was as described (Suresh et al., 2003). DMEM, trypsin-EDTA, penicillin-streptomycin, Blastidin, Actin monoclonal anti mouse antibody and FITC were purchased from Sigma. Cy3, Cy5 coupled anti-mouse and anti goat IgG was purchased from Chemcon, India. FBS was purchased from JRH Bioscience and pSilncer 1.0U6 was purchased from Ambion. ^{32}P dCTP were from BRIT, EcoRI and ApaI were purchased from MBI, Fermentas, India. Chemiluminiscence reagent was from Pierce chemical company.

METHODS

Cell culture

Chicken embryonic fibroblast cells (CEF cells) were maintained at 37^0C (5% CO_2) in DMEM medium as described in (Suresh et al., 2006).

Design and preparation of constructs

Construction of plasmids that contain DNA templates for the synthesis of siRNAs was done as described by (Ambion tech notes). Briefly, siRNA target sites were typically chosen by scanning an mRNA (National Center for Biotechnology Information access number: AAC59718). Sequence for AA dinculeotides, recording the 19 nucleotides immediately downstream of the AA, and then comparing the potential siRNA target sequences were analysed by BLAST research to ensure that they did not have significant sequence homology with other genes. Then the chicken MPR 300 sequence was put into double-strand hairpin siRNA insert frame (designed by Ambion): strand 1, 5'-N (19) (sense) TTCAAGAGA N (19) (antisense) TTTTTT-3' (53 bp); strand 2, 3'-CCGG N (19) (antisense) AAGTTCTCT N (19) (sense) AAAAAATTAA-5'(61 bp); two strands of oligonucleotides were separately synthesized (Sigma-Aldrich). The MPR 300 hairpin siRNA insert was made by

annealing the two oligonucleotide strands together. The annealing reaction was performed by mixing 2 µl of each oligonucleotide with 46 µl of annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM Mg-acetate) and incubating the mixture at 90°C for 3 min, followed by incubation at 37°C for 1 h. The vector pSilencer 1.0-U6 (Ambion) was digested with EcoRI and ApaI (MBI, Fermentas), and the hairpin siRNA was ligated into the vector following the manufacturer's instructions. The sequence of the insert was confirmed by automated sequencing and by analyzing the fragments generated from digestion with HindIII. These vectors produce a short hairpin RNA with the linker sequence (TTCAAGAGA) that forms a looped structure, the linker being processed by Dicer, to generate a MPR 300 specific siRNA.

CHK_siRNA_NSK_1 FOR pSilencer-1.0-U6—Ampicillin

Sense strand spacer anti-sense strand Terminal

5'-CAATATTGCGCCTTAAGTG**TTCAAGAGA**CACTTAAGGCGCAATATTGTTTTTT-3',

5'-AATTAAAAAACAATATTGCGCCTTAAGTG**TCTCTTGAA**CACTTAAGGCGCAATATTGGGCC-3'

Analysis of siRNA effect

Reduction of gene expression by a specific pSilencer 1.0-U6 construct can be investigated by transfecting the plasmid DNA into experimental cells using standard DNA transfection techniques, such as calcium precipitation methods. The siRNA effect can be assessed at both RNA and protein levels by immunoprecipitation, immunofluorescence, western analysis, and northern analysis after 48 hrs of post transfection.

Transfection and selection

CEF cells were grown to about of 60 to 80% confluency (in 6-cm–diameter Petri dishes) and then cotransfected with 10 µg of pSilencer 1.0-U6 and 1 µg of pEF6/V5-His TOPO vector (Invitrogen; the plasmid containing the blasticidin-selectable marker gene) by calcium precipitation method (Praveen kumar et al., 2007). Transfected cells were selected for blasticidin resistance (10 µg /mL).

Antibodies

MPR 300 localization in CEF cells was detected by affinity purified antibody to the goat MPR 300 protein obtained as described earlier (Suresh et al., 2003). Mouse lysosome-associated membrane protein (LAMP) 1 was detected using monoclonal anti-mouse LAMP1 antibody. Immunoprecipitation of α- fucosidase was done with antibody against *unio* α- fucosidase enzyme that was raised in our laboratory as described (Siva kumar et al., 2004). Arylsulfatse A was detected using a rabbit antiserum (Suresh et al., 2005; Waheed et al., 1982).

Western blot analysis

Cells were harvested with 1 ml of 25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, using a rubber policeman and centrifuged at 300 x g for 7 min at 4ºC. The cell pellet was homogenized in 0.2 ml homogenization buffer (25 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 µg/ml bacitracin and 20 µg/ml soybean trypsin inhibitor) for 10 min on ice followed by sonication for 15–20 s. The protein concentrations in cell lysates were measured using the Bradford method. Twenty micrograms of total protein/lane was separated on 7.5% sodium dodecyl sulfate (SDS) gels and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry

milk in TBS containing 0.05% Tween 20 for 1 h and then incubated overnight at 4ºC with corresponding protein specific antibodies (see materials and methods). After washing and incubating for 1 h at 22ºC with a secondary antibody conjugated with horseradish peroxidase, the membranes were washed and immunoreactive bands were visualized by chemiluminescence (Pierce Western blot chemiluminescence reagent). Relative levels of protein in the different lanes were compared by analyzing scanned images using the NIH IMAGE program. All studies were performed a minimum of three times using independent cultures.

Immunofluorescence using confocal microscopy

To observe the reduction of MPRs gene expression by a specific siRNAs CEF cells were analysed by confocal microscopy using goat MPR 300 antibody for the localization MPR 300 protein. After transfection with pSilencer 1.0-U6 [with DNA templates synthesis for siRNA], the cells were fixed in 4% formaldehyde in PBS buffer incubated at room temperature for 6 min, washed, and then permeabilized with 0.2% saponin in PBS for 6 min at room temperature. The cells were washed with PBS and blocked with 5% BSA in PBS for 30 min at room temperature. The cells were then incubated with LAMP1 antibody (1:100 dilutions) followed by MPR 300 antibody 2 µg/ml in 5% BSA in PBS for 1 h at room temperature. The cells were washed 5–6 times with PBS, blocked with BSA as above, and incubated with fluorescent tagged anti-rabbit IgG-Cy5 and anti-mouse IgG-FITC (Chemicon, India) for 1 h at room temperature. Finally the cells were washed extensively with PBS and observed under confocal microscope for localization of MPR 300.

Metabolic labeling of cells and immunoprecipitation

Metabolic labeling with [^{35}S] methionine and immunoprecipitation was done as described (Siva Kumar et al., 2002).

Quantification of mRNA in silenced and wild-type cells

MPR 300 gene specific mRNA level was observed by Northern blot analysis.

Northern blot analysis

Using gene specific primers and MPR 300 cDNA in PCR, the probe was prepared. This was used for Random Primer labeling: (HexaLabel DNA Labeling Kit MBI Fermentas). Total RNA was isolated from siRNA transfected CEF cells for the indicated times (0-12 hours described in results) using Qiagen kit protocol according to the instructions of the manufacturer. Northern blot analysis was done as described (Suresh et al., 2004). In brief, total RNA (15µg) from transfected cells was subjected to denaturing agarose (1%) gel electrophoresis and transferred to Hybond N membrane (GE). The membrane cross-linked for 45s in a UV cross-linker and incubated for 3 h at 42^0C in hybridization buffer with the synthesized probes labelled with [α-^{32}P] dCTP (50µCi). A labelled probe was denatured at 98^{o}C for 5 min before dilution with hybridization buffer. Hybridization was carried out at 42ºC overnight. Membrane was washed for 10 min at RT and 30 min at 62^{o}C with 20XSSC buffer containing 0.1% SDS followed by exposed to Kodak film overnight at -70^{o}C with an intensifying screen. Alternatively the membrane was exposed to phosphor imaging.

Lysosomal enzyme assays

Lysosomal enzyme activities in the secreted medium as well as in soluble fraction were determined as described previously using the corresponding p-nitro-phenyl derivatised chromogenic substrates (Ludwig et al., 1993; 1994).

Chromatography on MPR 46 and MPR 300 affinity gel

To characterize the level of M6P polypeptides intracellularly and those secreted out of the cells, the control cells, MPR 300 silenced cells and the scrambeled cells, were all separately metabolically labelled with [^{35}S] methionine as described above. After

labelling the medium containing the secretions was collected, and the soluble extract of the cells was prepared. These were then processed on a receptor-affigel. MPR 46 and MPR 300 were purified from goat liver as described and coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. The secreted media and soluble extract obtained after labelling with ^{35}S-methionine were subjected to MPR affinity chromatography as described (Braulke et al., 1990). The mannose 6-phosphate eluates were precipitated with 10% trichloroacetic acid; pooled and dissolved in 50 µl of 0.4 M Tris-HCl, heated for 5 min at 95 °C in 10 mM dithiothreitol, 1% SDS; characterized by SDS-PAGE (10%) and fluorography.

Endocytosis of M6P-containing [^{35}S] polypeptides

The M6P-containing [^{35}S] polypeptides were purified on affinity column containing a mixture of both MPRs concentrated by acetone precipitation at -80ºC and dialyzed against DMEM. siRNA treated, scrambled and control cells were incubated for up to 30 min with 0.5 ml MEM containing 50,000 cpm of purified M6P-containing [^{35}S] polypeptides. The cells were lysed as described above and after centrifugation for 30 min at 100xg, the supernatant and the pellet were counted in a scintillation counter.

RESULTS

Selection of siRNA transfectants: CEF cells were cotransfected with pSilencer 1.0-U6 generating short hairpin siRNA and with pEF6/V5-His TOPO expression vector carrying selectable blasticidin S marker as described in the materials and methods section. Transfected cells were selected for blasticidin S resistance and characterized by the presence of the insert in the genomic DNA (data not shown).

Analysis of MPR 300 protein expression

To confirm the role of MPR 300 in sorting and trafficking of lysosomal enzymes to lysossomes in non-mammalian vertebrates, further investigations were carried out through siRNA-mediated knockdown experiments involving down regulation of MPR 300. The siRNA sequences for MPR 300 were designed as per standard protocols. The cells were transfected with vector that can synthesize MPR 300 specific siRNA (Fig. 3.1) and the protein levels were analysed by immunoprecipitation. The results showed an efficient down regulation of the MPR 300 in chicken embryonic fibroblast cell lines. Scrambled sequences were used as negative controls (Fig. 3.2).

Immunofluorescence analysis with polyclonal anti-MPR 300 and polyclonal anti-LAMP1 antibody showed that MPR 300 fluorescent protein signal (red) of pSilencer siRNA–CHK-MPR cells was reduced by 98%, compared to control cells (Fig. 3.3B, lower panel). LAMP1 immunofluorescent signal (green) was similar in control and transfected cells (Fig. 3.3A).

Northern analysis of MPR 300

For the analysis of effect of siRNA, we did northern blot analysis using probe synthesized based on the chicken MPR300 gene. Analysis was performed after 48hrs of post transfection. From the fig.3.4 we can observe expression level of the MPR 300 gene specific mRNA. The expression was decreased almost below 5% at

60hrs of post transfection, lower panel is Et-Br stained gel picture, showing equal amount and quality of loading concentration of total RNA.

Immunoblotting analysis

To determine whether the expression of siRNA-CHK-MPR 300 affected MPR 300 protein production, constant protein amounts of lysate prepared from siRNA CHK-MPR 300 transfected cells were used to perform western blot analysis with polyclonal anti-goat-MPR 300 antibodies. A specific protein band (Mr = 300,000) was present in all the samples whose concentration was reduced in the cells below 5% at 60 hrs of post- transfection (Fig. 3.5A and B)

Effect of down regulation of MPR 300 on lysosomal enzyme sorting to lysosomes

The consequence of silencing the MPR 300 in CEF cells was observed by analyzing the intracellular and extracellular activities of five soluble lysosomal enzymes known to be transported to lysosomes via MPRs (β-hexosaminidase, arysulfatse A, α-fucosidase, α-mannosidase, α-galactosidase and cathepsin D) whose concentrations were measured after a 60 hrs of post transfection. Silencing resulted in a reduction of upto 7-10% of these enzyme activities when compared to the control and scrambled (Fig.3.6A). Under the same conditions the fraction of activity of these enzymes that accumulated in the secretions, increased to 72–93% in MPR 300 silencing fibroblasts when compared with control and scrambled (Fig.3.6B). These results suggest that the loss of MPR 300 by silencing results in missorting of lysosomal enzymes to lysosomes.

CEF cells silenced for MPR 300 was first tested for their ability to transport lysosomal enzymes to lysosomes. In this study we examined the levels of the M6P containing polypeptides intracellularly as well as those secreted out of the cells in

both control, scrambled and silenced after metabolic labelling of the cells. The secretions and soluble extract were passed over goat receptor-affinity gel. After extensive washing, bound ligands were eluted with Man-6-P and polypeptides analyzed by SDS-PAGE and fluorography. The results shown in this Fig 3.7A and B demonstrates that a number of Man-6-P containing polypeptides eluted from the receptor gel was very high in soluble fractions of control and scrambled cells (F.g.3.7 A, lanes 1and 2), while they were low in the cells silenced for MPR 300 (Fig.3.7 A ,lane 3) as these cells secrete a large amount of Man 6-phosphorylated ligands when compared to controls (Fig.3.7B lanes 3, 1 and 2). From these results, we conclude that, fibroblasts lacking CI-MPR expression missort a significant fraction of their newly synthesized lysosomal enzymes; indicating that in embryonic fibroblasts the MPR 300 is responsible for most of the intracellular transport of soluble lysosomal enzymes.

Determination of missorting of newly synthesized α-fucosidase in MPR300 silenced fibroblasts

To further study the effect of silencing MPR 300 in CEF cells, we also monitored the levels of the specific enzyme α-fucosidase by immunoprecipitation experiments. In silenced cells almost 80% of the enzyme was secreted out into the medium. Fibroblasts were metabolically labelled in the presence of [^{35}S] methionine for 1 h. Cells were then chased for 8 h to allow transport of labelled lysosomal enzymes to their final destination, α-fucosidase was immunoprecipitated from the cells and secretions. From fig.3.8, in the control cells, 0.4% of the labelled fucosidase was recovered in the secretions and 98.6% intracellularly, while in MPR 300 silenced fibroblasts, the percentages were 79.2% and 20.8%, respectively. This clearly

demonstrates that the bulk of newly synthesized enzyme is secreted into the culture medium due to the silencing of the MPR 300.

MPR 300 regulates endogenous arylsulfatse A localization

To determine MPR 300 is a trafficking receptor and to conform its role in intracellular transport of lysosomal enzymes to lysosomes in CEF cells, we investigated the possibility that the MPR 300 might be important for sorting of arylsulfatase A, we determined the effect of the MPR 300 silencing on endogenous arylsulfatase A sub cellular distribution. As shown in Fig. 3.9B, in the MPR 300 expressing control cells, CEF arylsulfatase A is present in early/late endosomes near the perinuclear region. In contrast, in the MPR 300 silenced CEF cells the level of endogenous arylsulfatase A dramatically reduced, similar to the pattern observed in MEF cells that lack both receptors. (Fig 3.9F). We did also observe the colocalization between arylsulfatse A and the lysosomal protein LAMP-1. This confirms the role of the MPR 300 in lysosomal enzyme sorting and trafficking.

Figure 3.1: Schematic representation of and construction of siRNA vector for RNAi mediated MPR 300 silencing in CEF cells

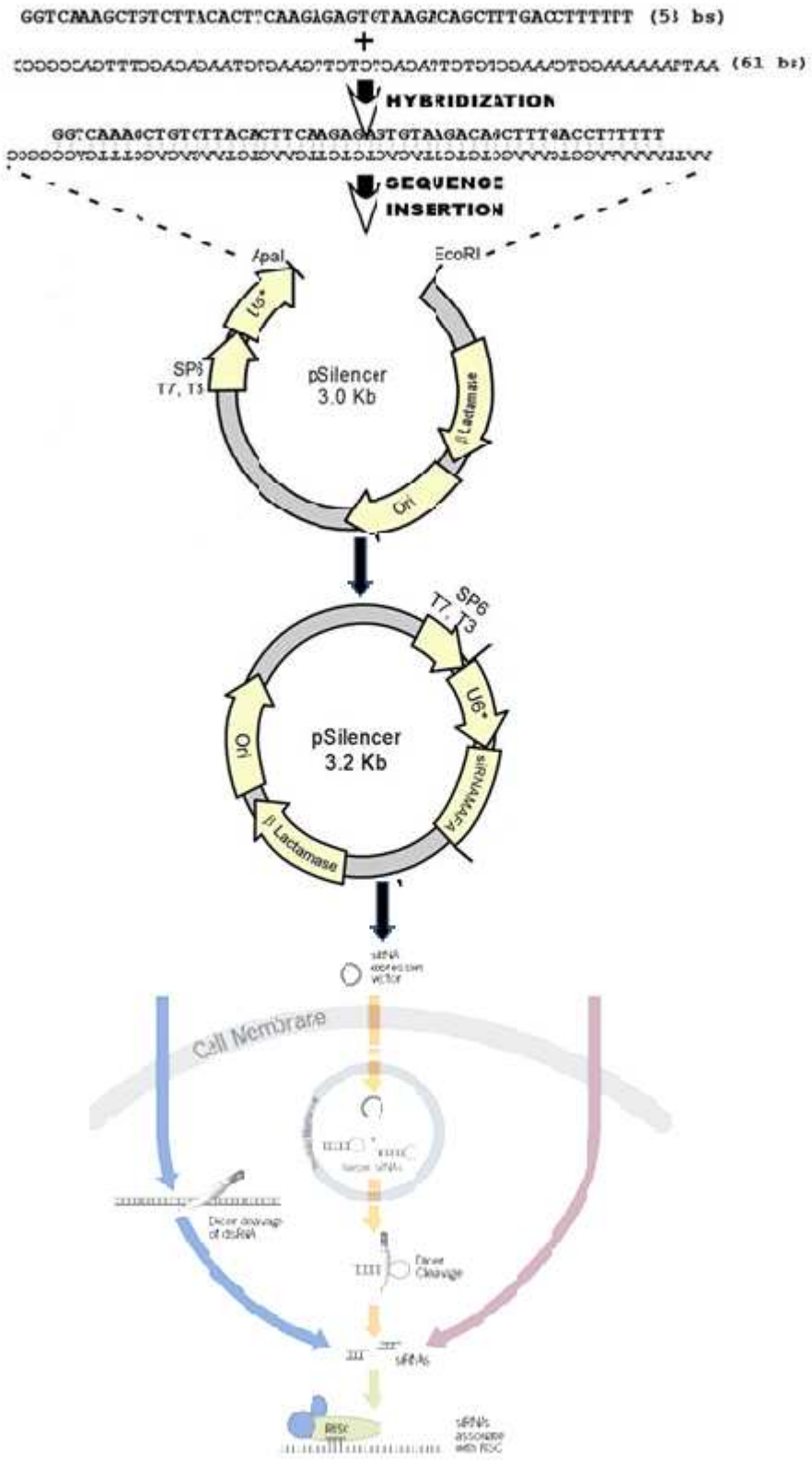

HYBRIDIZATION
SEQUENCE
INSERTION
ApaI
EcoRI
pSilencer
3.0 Kb
pSilencer
3.2 Kb
Ori
β Lactamase
U6+
T7, T3
SP6
Cell Membrane
RISC

Figure 3.1

Figure 3.2: siRNA mediated down regulation of MPR 300 in CEF cells. MPR 300 specific siRNA was synthesized by transfecting the psilencer-CHK-MPR 300-siRNA. The cells were incubated for 56 hrs. The down regulation of MPR 300 was analysed by Immunoprecipitation analysis. Lane 1 control, lane 2 is scrambled and lane 3 is siRNA treated cells.

Figure 3.3: RNAi mediated MPR 300 knockdown in CEF cells. To selectively silence MPR 300, CEF cells were stably transfected with either psilencer 1.0 U6 CHK-MPR 300 siRNA and selected with blasticidin S. Cells were grown on cover slips and the immunofluorescence staining of MPR 300 in the clone expressing MPR 300 RNAi was done as described in materials and methods. Nuclei were visualized using DAPI. CEF cell transfected with MPR 300 small interference RNA (siRNA) vector (sequence 1). The siRNA abolished the perinuclear staining of MPR 300 antibody (red). The overlay shows green fluorescence only, produced by the anti-LAMP1 antibody (a lysosomal membrane marker protein). Cell transfected with vector alone (control) immunostained with anti-LAMP1 (green) and anti-MPR 300 (red) antibodies. (*Bars.15μm*).

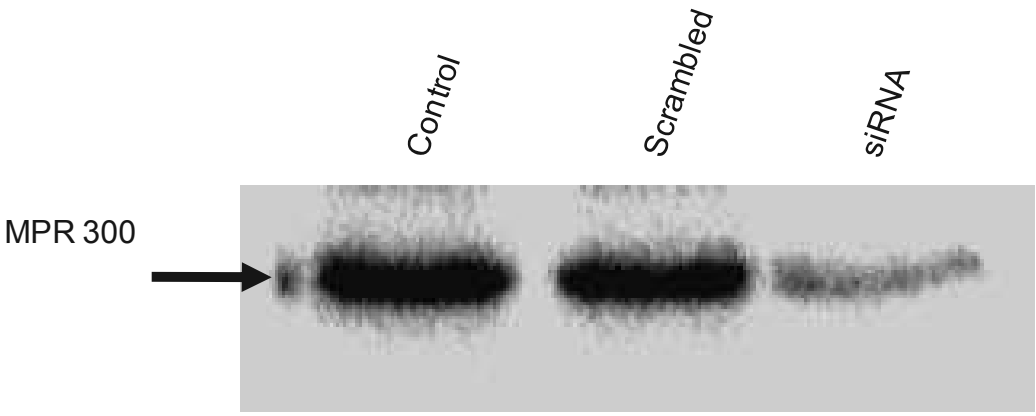


Figure 3.2

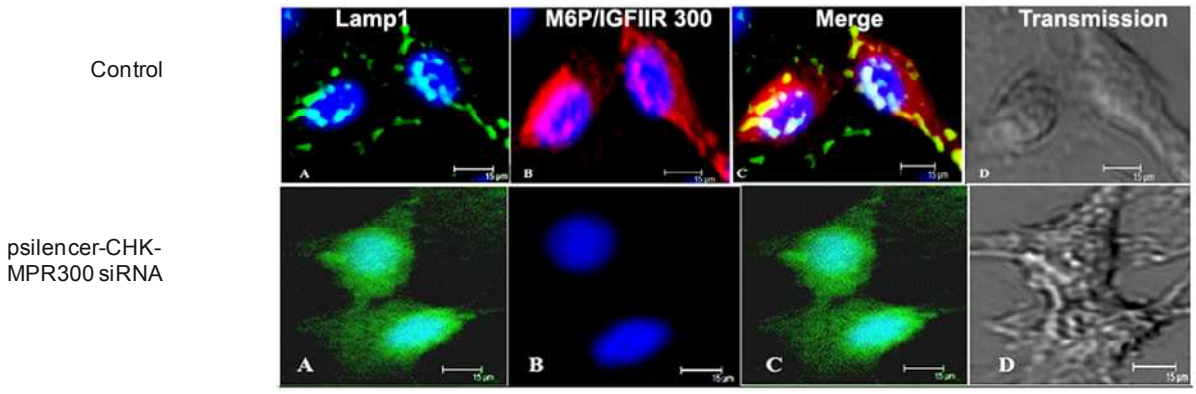
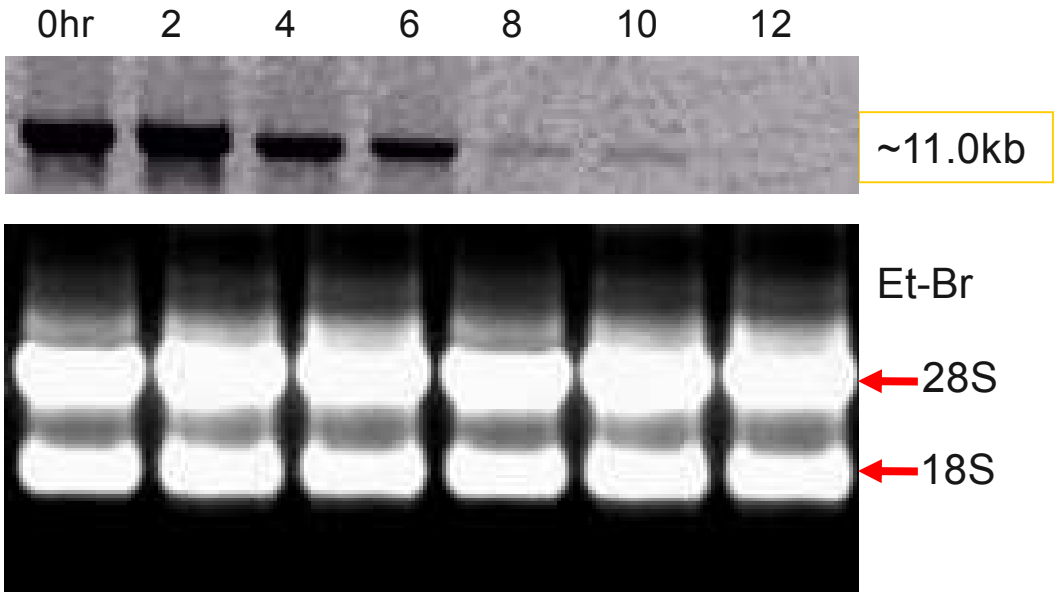
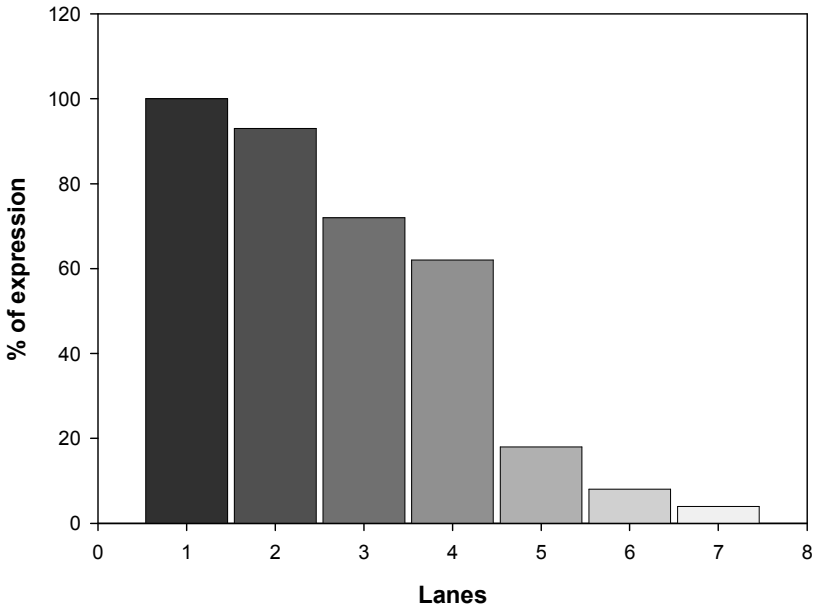


Figure 3.3

Figure 3.4: Expression of MPR 300 in CEF cells treated with pSilencer-CHK-MPR 300 siRNA. (A) Total cellular RNA was isolated from CEF cells treated with pSilencer-CHK-MPR 300 siRNA for the indicated times. Northern blot analysis was done using a cDNA probe to chicken MPR 300, whereas ethidium bromide stained gel was used as a loading control as described in materials and methods. (B) Bar graph showing decreasing level of MPR 300 mRNA


0hr
2
4
6
8
10
12
~11.0kb
Et-Br
28S
18S

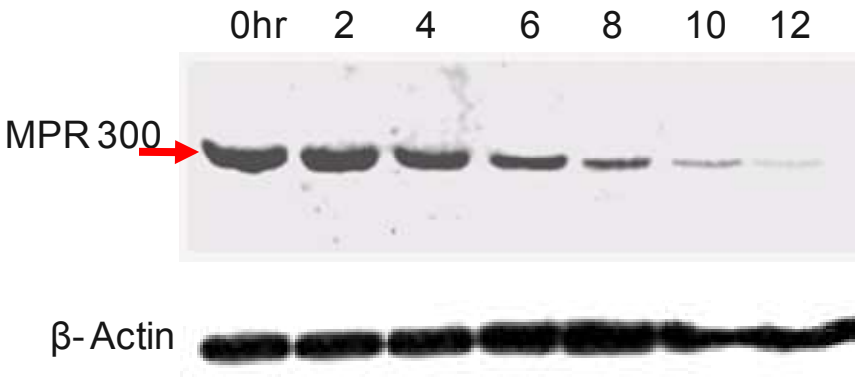
(A)


120
100
80
60
40
20
0
% of expression
0
1
2
3
4
5
6
7
8
Lanes

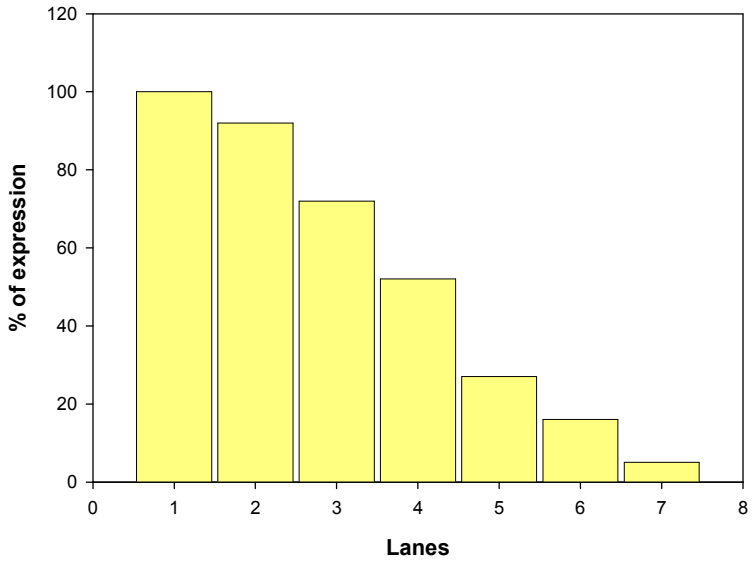
(B)

Figure 3.4

Figure 3.5: Silencing MPR 300 in CEF cells with RNAi. (A) Efficiency and specificity of siRNA targeted against MPR 300 pSilencer-CHK-MPR 300 and pEF6/V5-His TOPO vector were co-transfected into CEF cells. Cells were harvested 2 days after transfection and homogenized in lysis buffer and then analysed by immunoblotting with affinity purified goat MPR 300 IgG followed by horseradish peroxidase-conjugated antibody (upper panels) and anti-actin antibody (lower panels). Note that pSilencer-CHK-MPR 300 efficiently silenced the expression of MPR 300 (Upper panel) in CEF cells at 60hrs of post transfection. (B) Bar graph showing decreasing level of MPR 300 protein.


0hr 2 4 6 8 10 12
MPR 300
β- Actin

(A)

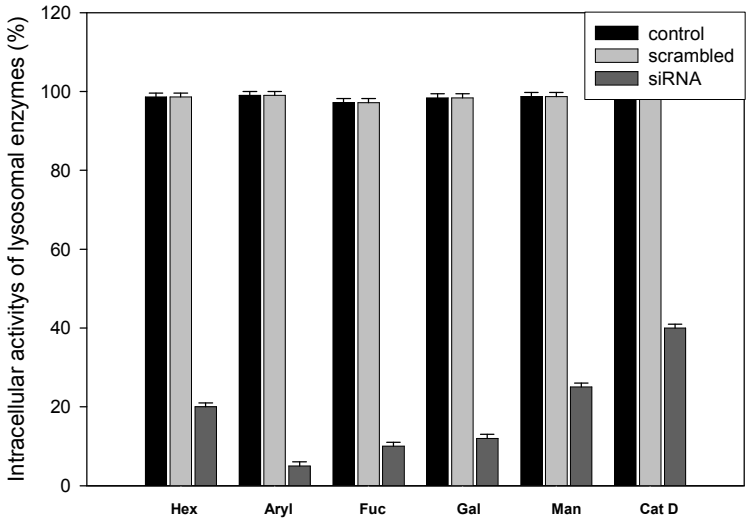

120
100
80
60
40
20
0
% of expression
0 1 2 3 4 5 6 7 8
Lanes

(B)

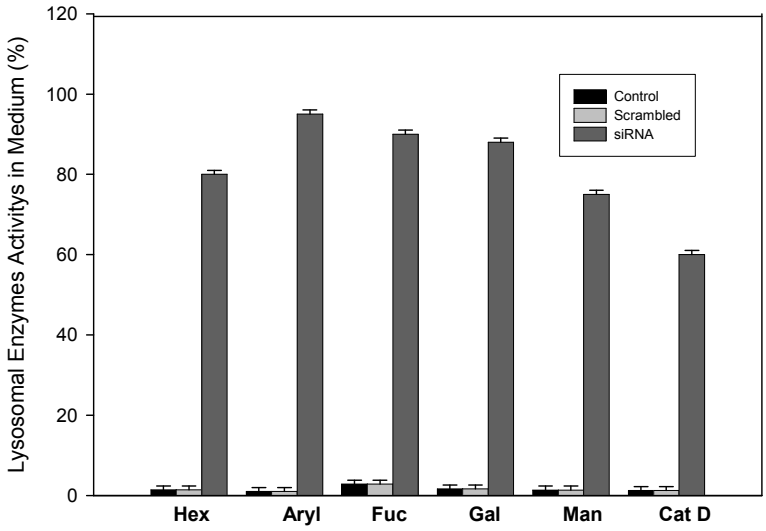
Figure 3.5

Figure 3.6: (A) Intracellular activity of lysosomal hydrolases in control and MPR-silenced chicken embryonic fibroblasts. The percentage of specific activity is represented as shown: The assays were done in duplicate; mean values are given.

B) Activity of lysosomal hydrolases in secretions of control, scrambled and MPR 300 silenced chicken embryonic fibroblasts. The activity of five lysosomal hydrolases was determined in the media, and the cells of chicken embryonic fibroblasts were collected after a 60 hrs culturing period as described under “materials and methods.” The *bars* give the activity measured in the secretions in percent of total activity. The assays were done in duplicate; mean values are given.


Intracellular activitys of lysosomal enzymes (%)
120
100
80
60
40
20
0
control
scrambled
siRNA
Hex
Aryl
Fuc
Gal
Man
Cat D

(A)


Lysosomal Enzymes Activitys in Medium (%)
120
100
80
60
40
20
0
Control
Scrambled
siRNA
Hex
Aryl
Fuc
Gal
Man
Cat D

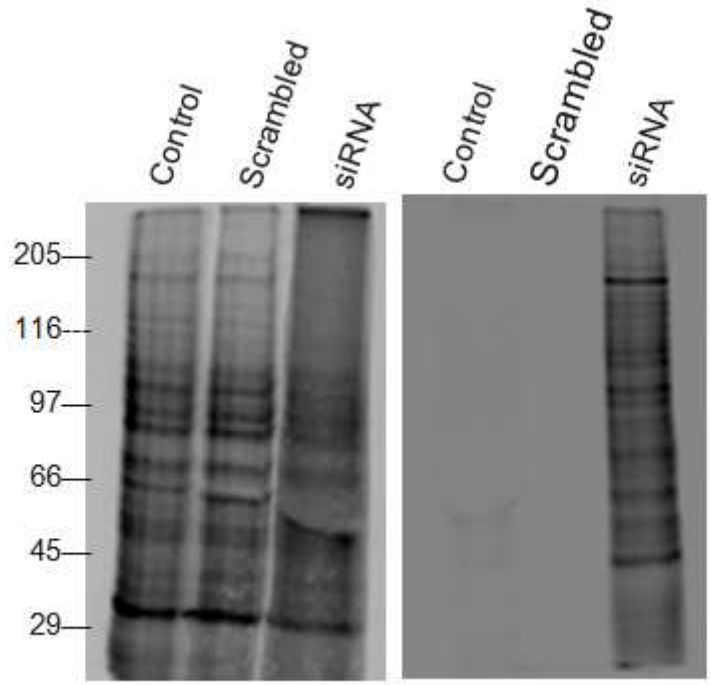
(B)

Figure 3.6

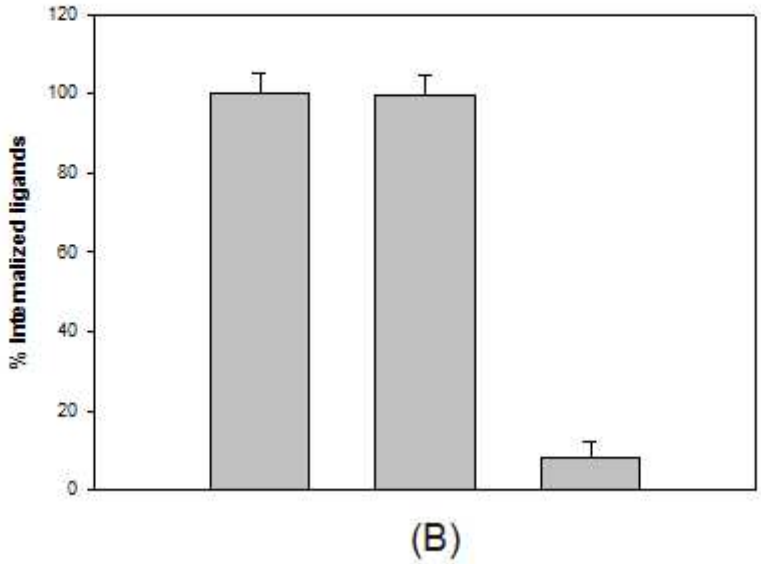
Figure 3.7: Binding of Man-6-P-containing polypeptides in soluble fraction as well as secretions of control, scrambled and MPR-deficient chicken embryonic fibroblasts to immobilized MPR 46 or MPR 300.

(A) **and (B).** Soluble fraction and **s**ecretions of metabolically labeled fibroblasts were passed over a MPR 46/ 300-Affi-Gel column, at pH 6.5. The material bound to the column and eluted with 5 mM M6P was separated by SDS-PAGE. Molecular mass standards (in kilodaltons) are given on the left. **I**n **A** and **B,** lane 1 and 2 are samples of control and scrambled ane 3 in both fig. is samples from silenced cells.

C) Effect of MPR 300 gene silencing on endocytosis of M6P-containing polypeptides. The [^{35}S] M6P-containing polypeptides were isolated from secretions of siRNA treated CEF cells by affinity chromatography on MPR 46/300 Affigel 10 and incubated for up to 30min with control, scrambled and MPR 300 silenced cells. The indicated numbers represent the percentage of internalized phosphorylated ligands.


Control
Scrambled
siRNA
Control
Scrambled
siRNA
205
116
97
66
45
29

(A)


120
100
80
60
40
20
0
% Intemalized ligands

(B)

Figure 3.7

Figure 3.8: Effect of gene silencing of MPR 300 on intracellular sorting of α-fucosidase in CEF cells. Immunoprecipitation of α-fucosidase. Equal amounts of protein were taken in each lane. The cells were labeled with [^{35}S] methionine for 1 h and then chased as indicated. α-fucosidase was immunoprecipitated from the cells **(C)** and culture medium **(M)**. The bands were quantitated by densitometry.

Figure 3.9: Intracellular localization of ASA in control fibroblast cells and siRNA treated cells. Control fibroblast cells (upper panel) and siRNA treated cells (lower panel) were transfected with psilencer-CHK-MPR 300-siRNA and stained with anti-ASA antibody followed by CY5 conjugated goat anti-rabbit IgG. The lysosomes of control fibroblast cells (A, upper panel) and siRNA treated cells (E, lower panel) were stained with anti- LAMP I antibody followed by FITC conjugated goat anti-mouse IgG. (C) is the merged image of (A) and (B). (G) Is the merged image of (E) and (F). Note that the intensity of the granular immunostaining for ASA in siRNA treated cells is decreased (red). (Scale Bar is *17- μm*).

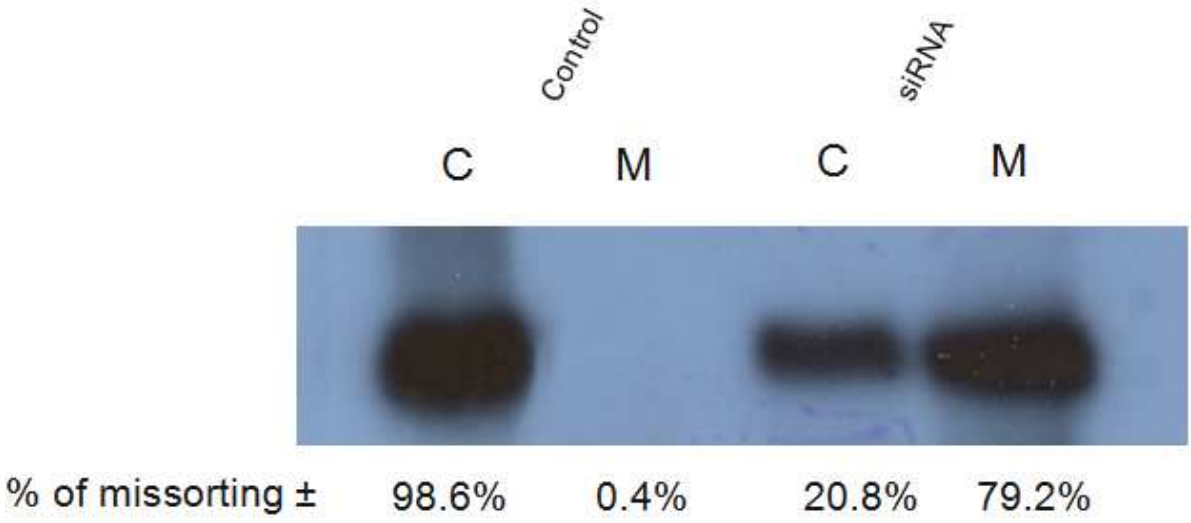


Figure 3.8

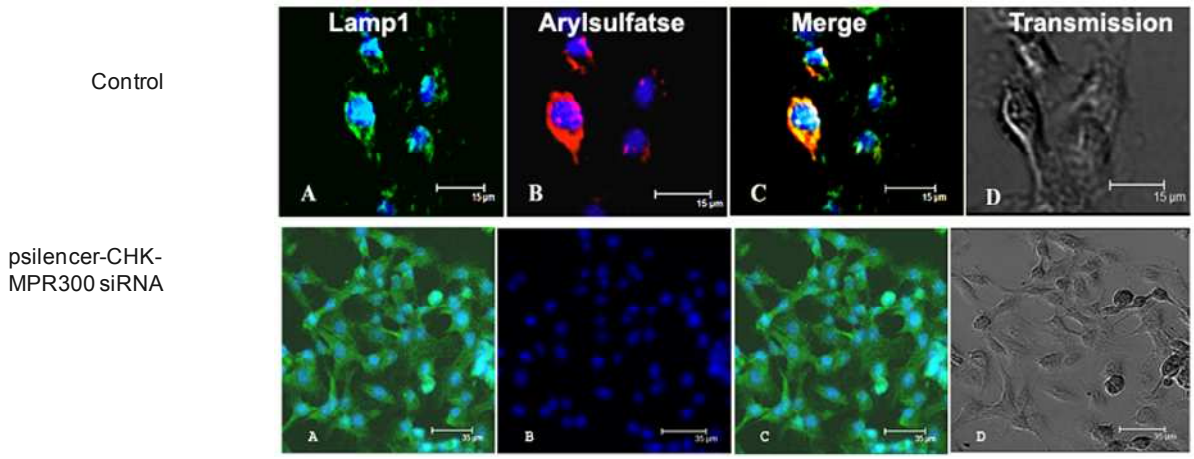


Figure 3.9

DISCUSSION

M6P-dependent transport of soluble lysosomal enzymes is a crucial step in the biogenesis of lysosomes. Newly synthesized lysosomal enzymes are carried to the lysosomes by vesicular transport from the endoplasmic reticulum, through the Golgi complex and endosomes. Initial transport steps are shared with proteins of the secretory pathway and apparently do not require specific signals. At the *trans* face of the Golgi complex, soluble lysosomal enzymes bind M6P receptors by their M6P-recognition signal and are subsequently transported via clathrin-coated vesicles to late endosomes (also termed prelysosomes) where the enzyme release is triggered by the acidic interior (von Figura and Hasilik, 1986). The enzymes are then transported to the lysosomes by capillary movement and M6P receptors are either targeted to the cell surface or carried back to the Golgi complex (Dahms and Hancock, 2002). In higher eukaryotes, the transport of soluble lysosomal enzymes involves the recognition of their mannose 6-phosphate signal by two receptors: the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR) and the cation-dependent mannose 6-phosphate receptor (CD-MPR). However, several lines of evidence suggest that the M6P/IGFII receptor is more efficient than the CD-M6P receptor in effectuating the intracellular sorting of newly synthesized lysosomal enzymes (Hille-Rehfeld et al., 1995). Other studies using gene-targeting technology defined the *in vivo* function of the MPRs. Mutant mice lacking the CD-MPR are viable and apparently have normal steady state levels of lysosomal enzymes (Ludwig et al., 1993; Koster et al., 1993). Mutant mice lacking the CI-MPR accumulate high levels of IGF-II and usually die prenatally (Ludwig et al., 1996; Lau et al., 1994; Wang et al., 1994). This is directly attributed to over stimulation by IGF-II, as CI-MPR-deficient mutants are completely viable in an IGF-II-

deficient background, with the double mutants having a dwarf phenotype similar to that of the single IGF-II-deficient mutants. Despite the viability of the single MPR-deficient mice, the importance of the MPRs in lysosomal enzyme targeting *in vivo* was indicated by the finding that triple mutants lacking the CD-MPR, CI-MPR and IGF-II are not viable (Ludwig et al., 1996). However, primary fibroblasts from embryos lacking both MPRs are almost totally impaired in intracellular lysosomal enzyme sorting and, as a consequence, accumulate undigested material in their endosomes/lysosomes (Ludwig et al., 1994). This phenotype, similar to that of I-cell fibroblasts characterized by a lack of synthesis of the Man-6-P signal on lysosomal enzymes, clearly illustrates the importance of the two MPRs as major components of the Man-6-P-dependent targeting system in fibroblasts. Our laboratory has isolated and purified the MPR proteins from mammals, different non-mammalian vertebrates and invertebrates, and showed that they exhibit similar biochemical and immunological properties compared to mammals (Siva Kumar et al., 1997; 1999; 2002; Udaya lakshmi et al., 1999; Raju et al., 2001; Dennes et al., 2005). So far, it has remained unclear why high eukaryotic cells express two different mannose 6-phosphate binding proteins. This can be answered only by comparative biochemical studies among the non-mammalian vertebrates, invertebrate species and specific cell lines. The comparative approach also helps to uncover the individual contributions of the two receptors to normal physiology and to pathological processes. Although both MPRs have been identified in CEF cells, its exact cellular localization and functions have not been extensively characterized like the mammalian proteins. To assess the role of MPR 300 in transport of lysosomal enzymes, in CEF cells, the present study was undertaken. In this study, we applied a DNA vector based CI-MPR 300-specific RNA interference approach to silence the

MPR 300 in CEF cells and to evaluate the biological consequences of this on lysosomal sorting. We designed DNA templates for synthesizing specific siRNAs based on chicken MPR 300 cDNA sequence to silence the CI-MPR 300 in CEF cells. In our system, the amount of MPR 300 protein, detected by immunofluorescence analysis, was shown to be lower in siRNA CHK-MPR 300 transfected cells. Our results are in line with what is known about the MPR 300 in mammals which has been described above. In our study, we observed that siRNA CHK-MPR 300–transfected cells were characterized by missorting of lysosomal enzymes.

From the present study we can conclude the following. It was found that specific silencing of MPR 300, but not of MPR 46, with siRNA significantly showed missorting of lysosomal enzymes in CEF cells. Since we have knocked down only the MPR 300 gene in these cells, the missorting of lysosomal enzymes was only to the extent of about 60–75% of the control cells, as the MPR 46 might have compensated for the transport of the remaining enzymes. Actually, it was found by co-immunostainings of LAMP1 and arylsulfatase A that almost 95% of the enzyme was secreted out into the medium in silencing cells. Since MPR 300 is also expressed at the plasma membrane of CEF cells, it is proposed that MPR 300 is the major receptor protein for the internalization of lysosomal enzymes from outside, through a M6P dependent manner in CEF cells. However this effect cannot be seen from our experiments in the MPR 300 silenced cells emphasising the role of the cell surface MPR 300 in internalization of the M6P containing ligands.

INTRODUCTION

Mannose 6-phosphate receptors (MPR 300 and 46) mediate transport of lysosomal enzymes to lysosomes in mammals. We purified homologous proteins from reptiles, amphibians, fish, and molluscs. Furthermore, our recent studies on the early vertebrate, fish receptors have conclusively established the evolutionary conservation of these proteins throughout the vertebrates. In the earlier chapters, the vertebrate receptors (MPR 46 and 300) have been well described. Although among the invertebrates, in the molluscs the receptors have been purified, no information is available whether the receptors are present in the highly evolved invertebrates, (echinodermates) which fall above the molluscs in the evolutionary tree. To establish the evolution of the receptor proteins in the invertebrates and in the animal kingdom, it is necessary to characterize the receptors in the highly evolved invertebrate species and we chose starfish species as the animals were kindly provided by Prof.Dr. S.Kelm from the University of Bremen. The present study was carried out to first identify the receptors from the starfish species, affinity purify and characterize them. Furthermore, since the MPR 46 protein is a cation dependent receptor in the vertebrates, we cloned, sequenced and expressed the starfish cDNA sequence in MPR deficient cell lines to study its function using various biochemical and immunological studies.

MATERIALS

Starfish animals were collected from Nord Sea, Germany. From some animals, the gonads were separated. Both the animals and gonads were kept frozen at minus 80 °C, and were kindly provided by Prof. Dr.Soerge Kelm, University of Bremen, Bremen, Germany. The whole animal tissue was used to prepare the acetone powder as described earlier (Siva Kumar et al., 1997). In some experiments, gonads were directly used. Affinity purified antibody to the goat MPR 300 protein was obtained as described earlier (Suresh et al., 2003). Antiserum to the purified α-fucosidase from mollusc was prepared as described (Siva Kumar et al., 2004). Phenyl Sepharose gel and Bicinchoninic acid were purchased from Sigma.

METHODS

Preparation of the total membrane extracts

The whole animal tissue was used to prepare the acetone powder as described earlier (Siva Kumar et al., 1997). All operations were carried out at 4 °C. 50 g of the acetone powder prepared was homogenized with 300 ml of 50 mM imidazole buffer pH 7.0, 150 mM sodium chloride containing 0.1 mM PMSF and the homogenate stirred overnight. The suspension was clarified by centrifugation at 13583 x g for 15 minutes, and the supernatant discarded. The pellet was homogenized with 300 ml of 50 mM sodium acetate buffer pH 5.0, containing 150 mM sodium chloride, and centrifuged as described above. The pellet obtained was finally homogenized with 300 ml of 50 mM imidazole-HCl buffer pH 7.0, containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride. To this Triton X- 100 and sodium deoxycholate were added to a final concentration of 1 and 0.1 % respectively and the suspension stirred overnight. This was then centrifuged at 670 x g, for 15 minutes, and the supernatant was recentrifuged at 32869 x g for 30 minutes. The

clear membrane extract was used as the source of the receptors. When the gonads were used, membrane proteins were directly extracted from them as described above for the purification of the receptors.

Affinity chromatography on phosphomannan – Sepharose gel

Phosphomannan–Sepharose (PM) gel was prepared as described earlier (Siva Kumar et al., 1997). To purify MPR 300, EDTA was added to the membrane extract to a final concentration of 2 mM, and applied on a PM gel equilibrated with column buffer (50 mM imidazole buffer (pH 7.0), containing 5 mM sodium β–glycerophosphate, 150 mM sodium chloride, 0.05 % Triton X-100, 2 mM EDTA (EDTA buffer). To purify MPR 46, $MnCl_2$, $CaCl_2$, and $MgCl_2$, were added to the membrane extract to a final concentration of 10 mM and applied to a separate PM gel equilibrated with column buffer described above except that the buffer had the three divalent metal ions in place of EDTA and no sodium chloride (metal ion buffer). This buffer allowed purification of the mollusc MPR 46 (Siva Kumar et al., 2002). The membrane extracts were passed through two separate phosphomannan-gels equilibrated with the EDTA and metal ion buffers for the isolation of the MPR 300 and 46, respectively. After the sample was passed through the gels, they were washed extensively with the respective column buffers, and the bound protein was eluted using 5 mM mannose 6-phosphate in column buffer. Aliquots were further analyzed by SDS-PAGE as described below.

Protein estimation

Protein estimation was done using bicinchoninic acid reagent following manufacturer's instructions.

SDS-PAGE and Western blot analysis

Samples were analysed by SDS-PAGE under non-reducing and reducing conditions as described (Laemmli et al., 1970). Gels were stained using the silver staining method (Blum et al., 1987). For immunological detection of MPR proteins, aliquots of the purified proteins were electrophoresed, and the proteins transferred to nitrocellulose membrane as described (Towbin et al., 1979). The membrane was placed in blocking solution containing 5 % Non-fat milk powder in Tris-HCl buffer pH 7.4, containing 0.15 M NaCl (TBS). For the detection of MPR 300, 10 µg of affinity purified goat MPR 300 IgG was used as the primary antibody. For the detection of MPR 46, antiserum raised against purified goat MPR 46 protein, (1 : 500 dilution) as well as 10 µg of the MSC1 antibody (affinity purified antibody obtained from the MSC1 antiserum raised against the cytoplasmic tail of the human MPR 46 protein) were used. Alkaline phosphatase (ALP) conjugated goat anti-rabbit IgG (1: 5000 dilution in TBS) was used as the secondary antibody. The blots were developed with BCIP-NBT substrate in ALP buffer and the bands visualized and photographed. Purified goat MPR 300, *unio* MPR 300 (invertebrate, mollusc) and goat MPR 46 were used as positive controls.

Isolation and identification of α-fucosidase enzyme in the starfish

Enzyme assay and partial purification of the enzyme was carried out as described (Siva Kumar et al., 2004). Acetone powder (50 g) of whole animal tissue or intact gonads (5 g) were separately homogenized with 4 volumes of 25 mM Tris-HCl buffer pH 8.0 (Buffer A). The homogenate was allowed to stir overnight to ensure complete extraction of the proteins and centrifuged at 17,226 x g for 30 min. The pellet was discarded and the supernatant was recentrifuged for 15 min. The extract was applied on a DE-52 ion-exchange gel (30 ml) pre-equilibrated with buffer A. The column was

washed with the same buffer until A_{280} reached 0.01. The bound protein was eluted as 10 ml fractions with buffer A containing 0.2 M NaCl (Buffer B). The peak fractions of DE-52 eluates containing protein and enzyme activity were pooled and $(NH_4)_2SO_4$ was added to give a final concentration of 1 M. This was applied on a phenyl-Sepharose gel (10 mL), pre-equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$ (Buffer C). The column was washed extensively with the same buffer and the bound enzyme activity was eluted in 5.0 mL fractions with buffer A. The active fractions from phenyl Sepharose gel were pooled and concentrated to 1.0 ml using Amicon concentrator (PM 10 membrane). When gonads were used, these were extracted as described above. The phenyl-Sepharose eluates were used in further studies.

Electro elution of partially purified α- fucosidase

The α-fucosidase band detected in SDS-PAGE in the phenyl-Sepharose eluates was electro eluted as follows. 250 µl of phenyl-Sepharose eluates were separated on 10 % SDS-PAGE followed by coomassie staining. The band corresponding to 56 kDa region was excised from the gel lanes, and the protein electro eluted. This protein was tested with antiserum to mollusc α-fucosidase in a western blot experiment, and also used for radio-iodination. Since the affinity gel fucosamine-Sepharose that is used to purify the enzyme is not commercially available the enzyme could not be affinity purified after the phenyl Sepharose chromatography and hence the band corresponding to the enzyme was electro eluted.

Iodination of α-fucosidase

Aliquot of the electroeluted sample was radio iodinated with ^{125}I-Na (MP Biomedicals, Rockford, IL, USA) using iodogen method as described (Siva Kumar et al., 2004) to a specific activity of 1 x 10^7 cpm / ug protein.

Immunoprecipitation

Aliquots of the radio iodinated enzyme were precipitated with trichloroacetic acid, the pellet was neutralized with 2 M Tris, and analyzed on 10 % SDS-PAGE to confirm the identity of the enzyme with respect to its molecular mass. For immunoprecipitation, 5, 00,000 cpm of the iodinated protein was taken separately into two tubes and incubated with preimmune serum and antiserum raised against the purified mollusc α-fucosidase enzyme (1: 1000 dilutions) overnight at 4°C in PBS containing 0.05 % Tween 20. The antigen-antibody complexes were adsorbed to protein-A-Agarose (40 µL of a 10 % suspension) (Bangalore genei, India). After incubation for 1 hr, protein-A-Agarose was collected by centrifugation and the pellet washed four times with PBS containing 0.05 % Tween 20. The immunoprecipitates were solubilized under reducing conditions and analyzed by 10 % SDS-PAGE. The gel was dried and the protein band detected by autoradiography.

Far-Western blotting of native α-fucosidase

Electro eluted α-fucosidase was electrophoresed in separate lanes on a 10 % SDS-PAGE and used in far-Western blot analysis as described (Siva Kumar et al., 2004). Bovine serum albumin (Sigma) was run as a negative control. After the electrophoresis, proteins were transferred onto to a polyvinylidene difluoride membrane. After blocking the membrane it was incubated with purified Starfish MPR 300 and with goat MPR 300 separately (100 µg) in PBS containing 1 % BSA, followed by detection with affinity purified goat MPR 300 antibody, followed by horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody.

RESULTS

Affinity purification of MPR proteins

The membrane extracts from the goat, chicken liver, and starfish were subjected to affinity chromatography on PM-Sepharose gel in the presence of 2 mM EDTA for the purification of MPR 300 alone. After washing the gel extensively, the bound protein was eluted with the column buffer supplemented with 5 mM mannose 6-phosphate. An aliquot of the eluted fractions were electrophoresed on 10 % SDS-PAGE in figure 4.1A lane 1 is goat MPR 300 and lanes 2 &3 are starfish MPR 300 from whole tissue and from gonads respectively. Consistent with our earlier findings (Siva Kumar et al., 1996) in all the species (mammalian, non-mammalian and in highly evolved invertebrates) the purified MPR 300 behaved as a single protein band corresponding to molecular mass of about 300 kDa. In addition to this the apparent Molecular mass of the non-reduced MPR 300 from both the species (Goat and the starfish) was nearly identical; to further characterize the purified MPR 300 from starfish in comparison to the mammalian receptor (goat MPR 300). We fractionated the protein on 7.5 % SDS-PAGE under reducing (+DTT) and non-reducing (-DTT) conditions where the protein showed a typical decrease in the electrophoretic mobility in the presence of DTT due to the openings of the disulfide bridges as the goat receptor showed similar property (Fig.4.1B).

For the purification of MPR 46 from goat and starfish, After depleting MPR 300 from the membrane extract, the flow through of PM-Sepharose gel was dialyzed extensively against the column buffer in the presence of $MnCl_2$ at a final concentration of 10 mM and applied on PM-Sepharose gel, pre-equilibrated with column buffer containing 10mM $MnCl_2$.After washing the gel extensively, the bound protein was eluted with the column buffer supplemented with 5 mM mannose 6-

phospate. An aliquot of the eluted fractions were electrophoresed on 10 % SDS-PAGE showing a bands at 46 region, in Fig. 4.1C of lane 1 is goat MPR 46, lane 2 and 3 the bands corresponds to MPR 46 from starfish.

Western blot analysis

Affinity purified goat, chicken and starfish MPRs were subjected to Western blot analysis to detect the specificity as described under methods. Affinity purified goat MPR 300 antibody can specifically detect goat MPR 300 protein (Fig. 4.2A lane1 and cross-reacts with starfish MPR 300, lane 3 and 4 are starfish MPR300, lane 2 is *Unio* MPR 300. Similarly, goat MPR 46 antibodies can also recognize both the goat and starfish MPR 46 proteins (Fig.4.2B). In addition to these, MSC1 antibody (peptide synthetic antibody, raised in the cytoplasmic region of human MPR 46) can also recognize both the goat and starfish MPR 46 proteins (Fig. 4.2C).

Purification of α-fucosidase from starfish whole tissue and from gonads

The clear supernatant of the crude extract obtained as described under the methods was applied on a Seralose-lactose affinity gel to deplete any lactose binding proteins from the extract (we identified some lactose-binding proteins from this animal species in our laboratory) and the flow through was applied on a DE-52 ion-exchange gel equilibrated with buffer A. After washing the gel matrix with buffer A, the sample was eluted using buffer B as described under methods. The elution profile was shown in fig.4.3A and the enzyme containing fractions were pooled, ammonium sulphate was added to a final concentration of 1 M, and this was applied on a phenyl Sepharose gel (hydrophobic affinity column) pre-equilibrated with buffer C. The bound sample was eluted using buffer A and the elution profile is shown in Fig 4.3B [From the total enzyme activity in the crude extract, only 59 % was

recovered in the DE-52 eluates, and from this 23.4 % activity was recovered in the phenyl-Sepharose eluates when the total animal tissue was used. When the gonads were used, 63 % and 41 % activities were recovered in the DE-52 eluates and phenyl Sepharose eluates respectively. The enzyme containing fractions was pooled and this was used for separation of the proteins and electro elution of the enzyme and lectin blotting.

Lectin blotting with biotinylated Con-A

To identify if the enzyme isolated in this study is a glycoprotein, to confirm its carbohydrate nature Con A lectin was used in ligand blotting. For detecting the enzyme, the phenyl Sepharose eluates was analysed with biotinylated Con-A in lectin blot, apart from other glycoprotiens a band appeared at 56 kDa region as shown in Figure 4.4. (Lane 1, eluate from whole animal tissue and lane 2 eluates from the gonads). It confirms the carbohydrate nature of the enzyme.

Immunoprecipitation and immuno blotting

The specificity of the antiserum raised against *unio* α-fucosidase enzyme for the electro eluted starfish enzyme was analysed by two independent methods, immunoprecipitation (using radio iodinated enzyme) and immunoblotting (using cold enzyme). In both conditions, the enzyme appeared as a single protein species with an apparent molecular mass of 56kDa that cross-reacted specifically with the antiserum raised against the *unio* α-fucosidase. Under the same conditions in immunoprecipitation, pre-immune serum did not show any reactivity with the iodinated enzyme suggesting the immunoprecipitation to be specific. The results of these are shown in Fig. 4.4B is immuno blotting lanes 1 and 2 is electro-eluted fucosidase from whole tissue and from gonads and in Fig.4.4C lane is

immunoprecipitation with *unio* fucosidase antiserum, lane 2 is with pre-immune-serum respectively.

Far-Western blotting of native α-fucosidase

Specific interaction of enzyme with the receptor was analyzed in a far-western blot assay. The electro eluted enzyme was separated on 10 % SDS-PAGE along with BSA and transferred to nitrocellulose membrane then it was probed with goat MPR 300 followed by incubation with affinity purified MPR 300 IgG which resulted in the visualization of bands that are shown in Fig 4.5A. Lane 1 of figure 4.5A showing the band is from fucosidase from whole tissue, lane 2 is from gonads and lane 3 is BSA where no band was recognized, similar results was found by probing the membrane with purified starfish MPR 300 as shown in Fig. 4.5B. It suggests that the interaction between receptor and the enzyme is specific.

Figure 4.1: SDS-PAGE analysis

A). 10 % SDS-PAGE analysis of the purified starfish MPR 300 protein. Lane 1, Molecular weight marker, lane 2 goat MPR 300 (positive control), lanes 3 and 4 represent starfish MPR 300 proteins from whole tissue and from gonads respectively. Arrow indicates the position of the receptor.

B). 7.5 % SDS-PAGE analysis of the purified goat (lanes 1 and 2) and starfish (lanes 3 and 4) MPR 300 proteins. Lanes 1 and 3 under non reducing conditions and lanes 2 and 4 under reducing conditions.

C). 10 % SDS-PAGE analysis of the purified goat and starfish MPR 46 protein. Lane 1, molecular weight marker, lane 2 goat MPR 46 (positive control) lanes 3 and 4 are starfish MPR 46 from whole tissue, and from gonads.

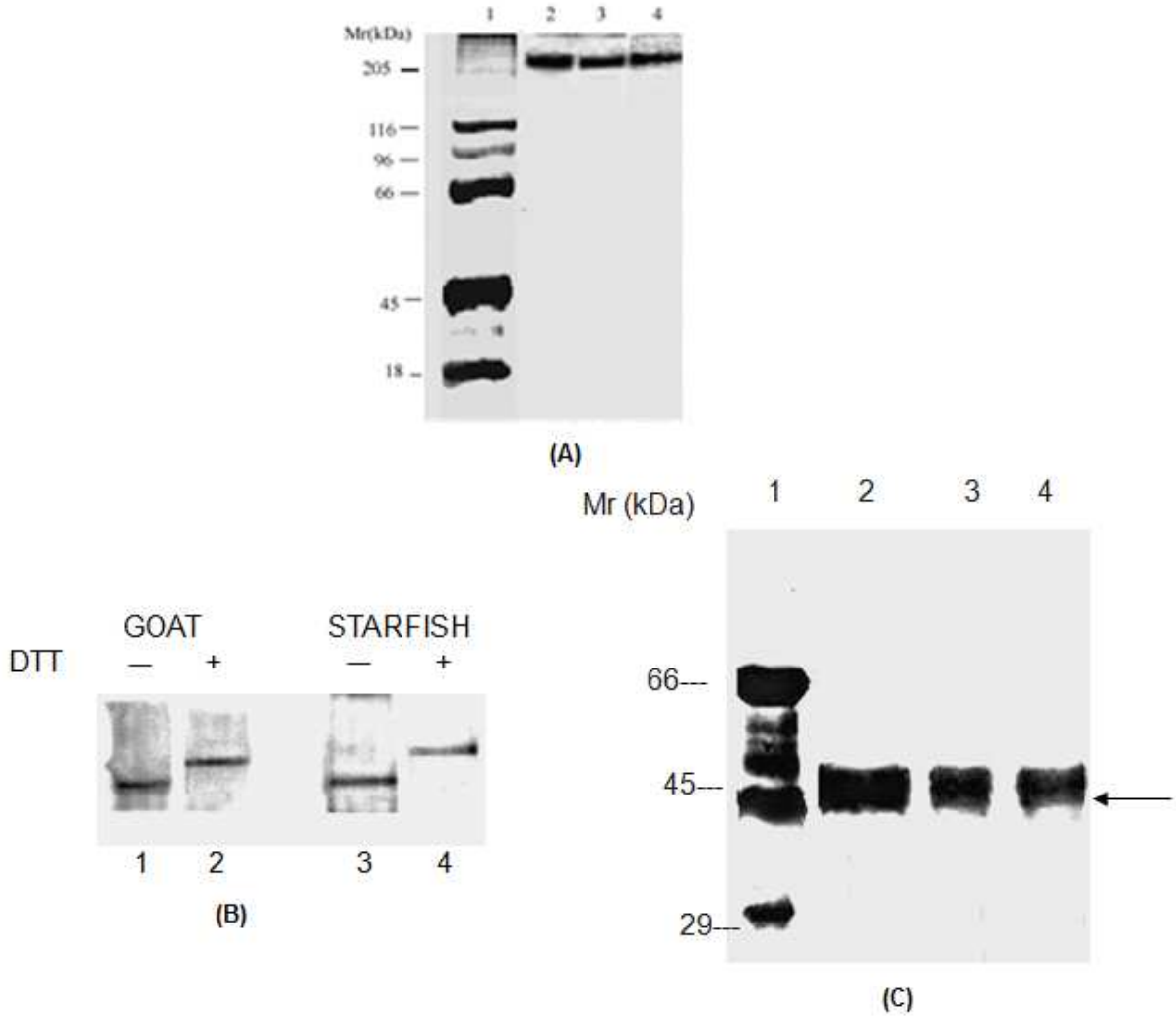

Mr(kDa)
205
116
96
66
45
18
1 2 3 4
(A)
DTT
GOAT
STARFISH
— + — +
1 2 3 4
(B)
Mr (kDa)
1 2 3 4
66---
45---
29---
(C)

Figure 4.1

Figure 4.2: Western blot analysis

A). Western blot analysis of starfish MPR 300 protein (10 % SDS-PAGE under reduced conditions). Lanes 1 and 2 represent purified goat and unio MPR 300 protein (positive controls), lanes 3 and 4 are starfish MPR 300 from whole tissue and from gonads, detected using affinity purified goat MPR 300 antibody. The arrow indicates the position of the MPR 300 protein.

B). Western blot analysis of goat and starfish MPR 46 proteins (10 % SDS-PAGE) detected using goat MPR 46 antiserum. Arrow indicates the position of the MPR 46 protein. Lane 1 goat MPR 46, (Additional band seen above represents the dimeric form of the receptor. Lane 2 and 3 are Starfish MPR 46 from whole tissue and from gonads.

C). Western blot analysis MPR 46 detected using affinity-purified MSC1 antibody. Arrow indicates the position of from whole tissue and from gonads.

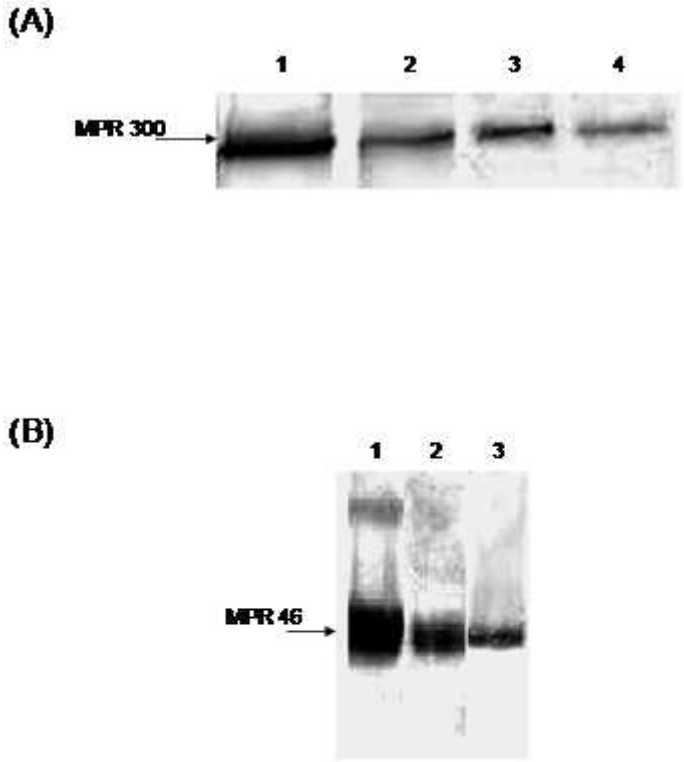
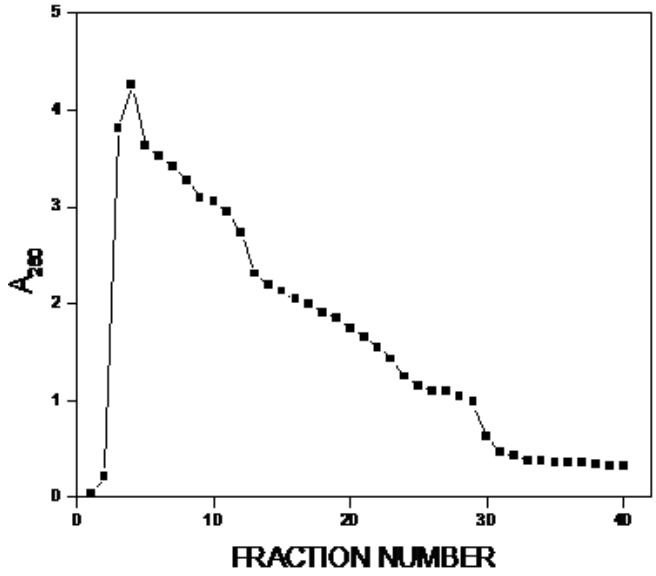


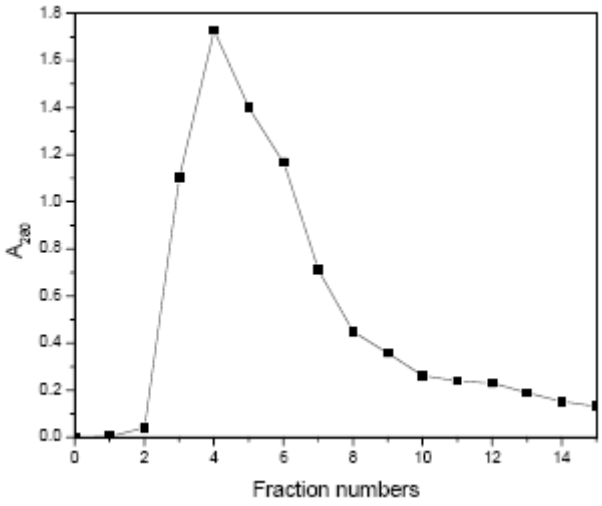
Figure 4.2

Figure: 4.3A. Elution profile of the protein from DE-52 (Anion-Exchange Chromatography).

Figure: 4.3B. Elution profile of the protein from phenyl-Sepharose gel. The active fractions from DE-52 gel were pooled and applied on phenyl-Sepharose gel and eluted as described under methods. Fractions 3-8 showing enzyme activity were pooled and processed further as described in the text.



(A)



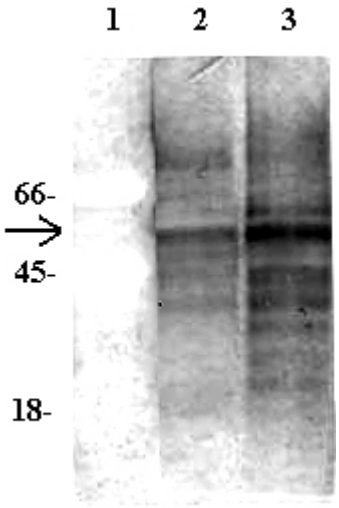
(B)

Figure 4.3

Figure: 4.4 (A) Lectin blotting. 10 % SDS-PAGE (under reduced conditions) analysis of α- fucosidase enzyme (Con-A blotting). Lane 1, molecular weight markers, lane 2 proteins from whole animal tissue eluted from the phenyl-Sepharose gel, lane 3, protein from the gonads eluted from phenyl-Sepharose gel.

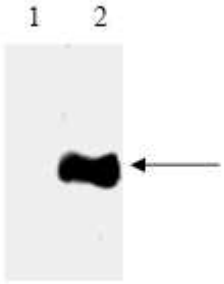
(**B)** Western blot analysis of the electroeluted α-fucosidase (details given under text). Lanes 1, 2 are enzyme from whole tissue and from gonads respectively.

(**C)** Immunoprecipitation of radiolabled α-fucosidase. Lane 1, pre-immune serum and lane 2 immune serum.



(A)

(B)



(C)

Figure 4.4

Figure: 4.5 (A) Far-western blot analysis of the electroeluted α-fucosidase enzyme with purified goat MPR 300 protein. Lanes 1, 2 represent enzyme from whole animal tissue and from gonads; Lane 3 is BSA (negative control).

B). Far-western blot analysis of the electroeluted α-fucosidase enzyme with purified Starfish MPR 300 protein. Lanes 1, 2 represent purified enzyme from whole animal tissue and from gonads. Arrow indicates the position of the enzyme.

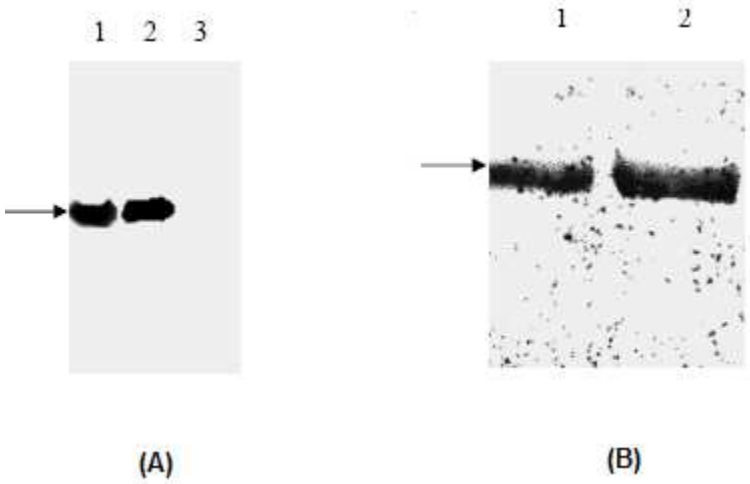


Figure 4.5

DISCUSSION

It is well established that the mammalian MPR proteins (MPR 300 and 46) mediate transport of lysosomal enzymes to lysosomes. Work from this laboratory provided the first biochemical and immunological evidence for the existence of mammalian homologues of these proteins from goat, reptiles, amphibians, and fish. Very little is known about how these proteins evolved and where in the animal kingdom they first started appearing. Work carried out in the past couple of years established that mammalian homologues of MPR proteins are present in the liver tissues and in specific cell types of different non-mammalian vertebrates (Siva Kumar et al., 1999; Udaya Lakshmi et al., 2000). A recent study further demonstrated that both MPR proteins are also present in the invertebrate molluscs (Siva Kumar et al., 2002). However prior to this study no information was available whether the receptors are present in the highly evolved invertebrates (star fish). Therefore the present study was undertaken in order to identify the putative receptors in starfish. The results of our experiments indicate that the starfish do contain the putative MPR proteins. Mammalian antibodies (goat MPR 300 antibody and MSC1 antibody (raised against a synthetic peptide in the region of cytoplasmic tail of human MPR 46, provided by Prof. Dr.K.von Figura, Goettingen, Germany) can recognize the MPR 300 and 46 proteins from starfish. It is interesting to note from the present study that the starfish MPR 300 protein also binds phosphomannan gel in presence of EDTA, and the MPR 46 protein can bind to the same gel in the presence of divalent metal ions. The bound proteins can be easily eluted using 5 mM mannose 6-phosphate and not by glucose 6-phosphate and therefore the binding is mannose 6-phosphate dependent. In this property the starfish proteins resemble the mollusc proteins which we have already characterized (Siva Kumar and von Figura, 2002).

Our previous experimental findings [biochemical, immunological and molecular cloning experiments from our lab] suggest that the putative MPR proteins with phosphomannan binding abilities are highly conserved throughout the vertebrates and also among the invertebrates. Below the molluscs, in Arthopoda and Annelidae we could identify only MPR 300 like polypeptides (Raju et al., 2001).

When the soluble extract of the starfish whole body animal tissue and the gonads were separately analysed for the α-fucosidase enzyme activity, we could detect the enzyme activity in both the samples. The rationale for using the gonads for the extraction of the enzyme was that in another invertebrate species, molluscs, it was shown that the activity of the enzyme is high in the gonads and the enzyme may play a critical role during the fertilization process. Furthermore we have already characterized the mollusc enzyme (Siva Kumar et al., 2004). In SDS-PAGE this showed a band corresponding to 56 kDa, and was also cross-reacting with α-fucosidase enzyme antiserum raised against the purified mollusc enzyme (Siva Kumar et al., 2004). In a separate study we have also detected that in the other invertebrates such as the arthropods (prawn) α- fucosidase enzyme activity is seen and the antiserum to the mollusc enzyme can cross-react with the enzyme from arthropods (unpublished information). Our results thus suggest that at least in three different invertebrate species contain antigenically similar α-fucosidase enzyme. Our lab already established and provided the first evidence that the putative receptors are present in the invertebrate mollusc with biochemical and immunological properties similar to the vertebrates (Siva Kumar and von Figura, 2002). The present results on the starfish protein, substantiates that the putative receptors are conserved among the echinodermates and molluscs.

Among the invertebrates we earlier identified and purified the putative receptors from the molluscs (Udaya lakshmi et al., 1999; Siva Kumar et al., 1999; 2002). Below the molluscs in the *Drosophila melanogaster,* only a lysosomal enzyme receptor protein (LERP) could be identified by us. This shows partial homology to the human MPR 300 protein but fails to bind on the multivalent phosphomannan gel. Furthermore unlike the mammalian and other known putative MPR 300 proteins that exhibit a molecular mass of 300 kDa, LERP shows a molecular mass of 150 kDa, and possibly represents a truncated form of the mammalian homologue. LERP contains five repeating units in its luminal domain that display a homology of 23-29.5 % at the amino acid level to those of the human MPR 300. However the residues known for mannose 6-phosphate binding are lacking in the LERP (Dennes et al 2006). No MPR 46 homologue is known in the *Drosophila.* Additionally in other species, such as the prawn (Arthropoda) and earthworm (Annelidae) also, only MPR 300 like proteins could be detected and no MPR 46 protein could be detected in these species (Raju et al., 2001). Table 4.1 describes the current knowledge about the MPR 300 proteins in the invertebrates.

In summary, from our results we can conclude the following: i) The highly evolved invertebrates, starfish contains the putative MPR proteins (MPR 300 and 46), that can be affinity purified on the multivalent Phosphomannan gels, like the mammalian and non-mammalian vertebrate receptors, ii) The biochemical and immunological properties of the starfish receptors resemble the mollusc, mammalian and other receptors characterized, iii) Additionally, a glycosylated α-fucosidase resembling the mollusc enzyme was also identified and characterized from the starfish. These data suggest that possibly starfish might also use a similar targeting pathway as the molluscs for the targeting of their lysosomal enzymes. Table 4.1 summarizes the

current knowledge of the MPR proteins among the invertebrates. Isolation of cDNA clones for the starfish and the mollusc receptors, and making a structural comparison of their sequences would throw further light on the conservation of the ligand binding domains of the receptors among the invertebrates. These studies would eventually lead to establish the evolution of the receptor proteins in the animal kingdom, particularly in the invertebrates, which is the future direction of work in our laboratory.

Table 4.1: Current knowledge about the MPR proteins in the invertebrates

Invertebrates Reference	Species	M6P dependent Binding to MPR's		Reactivity with mammalian PM gel MPR's		Molecular cloning MPR's		antibodies	
		300	46	300	46	300	46		
Invertebrates									
Echinodermata Present study	**Star fish**	**+**	**+**	**+**	**+**			**ND**	**ND**
Mollusca	**fresh water mussel**	**+**	**+**	**+**	**+**	**ND**	**ND**		**[1]**
Arthropoda	**prawn**	**@**	**#**	**#**	**#**	**ND**	**---**		
Annelidae	**Earthworm**	**@**	**#**	**#**	**#**	**ND**	**---**		**[2]**
Drosophila Genome	***Drosophila melanogaster* LERP (Mr 150 kDa)**	**No binding**	**##**	**ND**	**---**	**YES**	**---**		**[3]**

Star fish: *Asterias rubens* , fresh water mussel: ***Unio***, Prawn: ***Penaeus indicus***., Earthworm: ***Perionyx excavatus.,*** @ Only MPR 300 like proteins detectable***.,*** ND : Not determined***., #*** : Not detectable***.,***

[1] Siva kumar N, von Figura K (2002) Identification of the putative Mannose 6-phosphate receptor (MPR 46) protein in the invertebrate mollusc. Bioscience Reports, 22 5 & 6 513-521

[2] Raju VSN, von Figura K, Siva Kumar N (2001) Mannose 6-phosphate receptor (MPR 300) like polypeptides in the (Arthropods and Annelidae) Glycoconjugate J 18 29 C2 10

[3] Dennes A, Cromme C, Koduru S, Nadimpalli SK, Eble JA, Hahnenkamp A Pohlmann R (2005) The novel Drosophila Lysosomal Enzyme Receptor Protein mediates lysosomal sorting in mammalian cells and binds mammalian and Drosophila GGA adaptors J Biol Chem 280:12849-12857

INTRODUCTION

Mammalian mannose 6-phosphate receptors (MPR 300 and 46) mediate transport of lysosomal enzymes to lysosomes. Studies carried out by a number of researchers using molecular biology techniques, allowed cloning and sequencing of the MPR 46 and 300 proteins from different vertebrates. Structural comparison of the sequences with the known mammalian receptor proteins have given important informations on the conservation of the domain structures and ligand binding sites among the vertebrate receptors. Recent studies using fish (early vertebrate) as a model organism clearly established that the receptors are conserved throughout vertebrates (fish to mammals). The extent of such studies in the invertebrate species is very limited. Although we purified the mollusc receptors and identified only a lysosomal enzyme receptor protein (LERP) in the *Drosophila melanogaster* little is known about their structure and functional roles in the invertebrates.

Towards understanding the structure-function of the invertebrate receptors, we have in this study characterized the starfish receptors and found them to be exhibiting similar biochemical and immunological properties as the goat receptor. To gain new insights into the sequence of these receptors, and to understand their structural relatedness to the vertebrate proteins, we have chosen to clone and characterize the starfish MPR 46 gene in this study as it is relatively a smaller protein compared to the MPR 300.

Studying the function of the cloned starfish MPR 46 receptor gene can be accomplished by expressing the cDNA into receptor deficient cell lines and analyze if the protein is expressed and behaves like the putative receptor. The present study describes the expression, functional characterization of the starfish MPR 46 cDNA in MPR deficient mouse embryonic fibroblast cells.

MATERIALS

Goat MPR 300 antibody was as described in part A of this chapter. DMEM, trypsin-EDTA, penicillin-streptomycin, Blasticidin, Actin monoclonal anti mouse antibody and FITC were purchased from Sigma. Cy3, Cy5 coupled anti-mouse and anti goat IgG was purchased from Chemcon, India. FBS was purchased from JRH Bioscience and pcDNA6V5/His was purchased from invitrogen. ^{32}P dCTP were from BRIT, EcoRI, HindIII and XhoI were purchased from MBI, Fermentas, India. Chemiluminescence reagent was from Pierce chemical company

METHODS

Isolation of total RNA from starfish gonads

Total RNA from starfish gonads (20mg) was isolated with the RNeasy kit (Qiagen) according to the manufactures instructions, purity and integrity of the total RNA was analyzed by agarose (1%) gel electrophoresis under denaturation conditions.

ReverseTranscriptase-Polymarase Chain Reaction

From the isolated total RNA cDNA was synthesized by Reverse Transcription (MBI Fermentas, India) following the kit protocol with oligo (dT) as the primer. The cDNA synthesized was used as template for amplification of MPR 46 gene using the following degenerative primers designed based on the conserved sequences of other species MPR 46 protein. The primers are; sense primers ('5-GTGCTGGTSAGTGAATCYTAGG-3'), ('5- ATGCTGAACAGTGTAAGG -3') and anti sense primer ('5-CGTTCGGTAGYAARTGRTGATC-3') ('5-CATCGGTAGCAAGTGATC-3'). The MPR 46 gene was amplified by PCR with Taq polymerase and 12 pmoles of the respective forward and reverse primers with the following conditions (95^{o}CX15';94^{o}CX1';55^{o}C X 1'; 72^{o}CX 1'; for 32 cycles).

Molecular cloning of MPR 46

The PCR product was analyzed on 1% agarose gel electrophoresis. The single band obtained was excised; gel purified and subjected to TA cloning into pTZ57R vector (MBI Fermentas).The positive clones were selected by blue-white selection and the plasmid DNA was isolated by mini kit (MBI Fermentas). The size of the insert was confirmed by restriction digestion of the plasmid DNA with EcoRI and Hind III enzymes.

Northern blot analysis

Total RNA (15µg) from starfish gonads was subjected to denaturing agarose (1%) gel electrophoresis and transferred to Hybond N membrane (GE). The membrane cross-linked for 45s in a UV cross-linker and incubated for 3 h at 42^0C in hybridization buffer. cDNA fragment of MPR 46 (819bp) was radiolabled with α-^{32}pdCTP (50µCi). Labeled cDNA fragment was denatured at 98^{o}C for 5 min before dilution with hybridization buffer. Hybridization was carried out at 42ºC overnight. Membrane was washed for 10 min at RT and 30 min at 62^{o}C with 20XSSC buffer containing 0.1% SDS followed by exposed to Kodak film overnight at -70^{o}C with an intensifying screen. Alternatively the membrane was exposed to phosphor imaging.

DNA sequencing

DNA sequencing was done at Biosereve sequencing Pvt.Ltd. Sequence comparisons were performed with the CLUSTAL W method available online at http://www.justbio.com.

Construction of expression vectors

The 819 bp full length starfish MPR 46 cDNA that was originally cloned into pTZ57R vector was amplified by PCR using sense (Forward (5'-CG G*AATTC **ATGCTGAACAGTGTA**-3') and anti-sense (Reverse 5'- CCG C*TCGAG *TCA*

CATCGGTAGCAA-3') primers and sub cloned into mammalian expression vector pcDNA.6/V5-His A (Invitrogen) as described in (Praveen Kumar et al., 2007). The sequences shown in bold can anneal with the amplified fragment. * denotes the cleavage site and the stop codon sequence is shown in italics.

Cell culture and transfection

Mouse embryonic fibroblasts [mpr (−/−) MEF] were grown in Dulbecco's minimal essential medium supplemented with Glutamax-I (Gibco/Invitrogen) and 10% fetal calf serum (FCS). Transfection of MEF cells deficient in MPR 46 and MPR 300 [mpr (−/−) MEF] with the 20 µg of pcDNA.6/V5-His A containing the starfish MPR 46 gene by Calcium precipitation as described (Praveen Kumar et al., 2007). Stable cell lines expressing the starfish MPR 46 were selected in Blasticidin S antibiotic containing medium with the final concentration of 15 µg/ml. The selection medium is changed for every 3 days.

Antibodies

Starfish MPR 46 from expressed MEF cells was detected by goat 46 antiserum, MSC1 and 218 cytoplasmic tail antibodies. Mouse lysosome-associated membrane protein (LAMP) 1 was detected using monoclonal anti-mouse LAMP1 antibody. Cathepsin D, Arylsulfatse A and Hexosaminidase was detected using a rabbit antiserum (Suresh et al., 2005; Waheed et al., 1982).

Preparation of cell lysate and extraction of membrane proteins from the transfected cells

The cell lysate preparation and membrane protein extraction from starfish MPR 46 expressing and mock transfected (vector alone) mpr (-/-) MEF cells was prepared as described (Praveen Kumar et al., 2007).

Purification of starfish MPR 46 protein

The expressed starfish MPR 46 protein from stabelly transfected cell lines was purified by affinity chromatography on phosphomannan gel (PM gel), as described above in presence of metal ions. The wash fractions were collected and elution was performed with 5 mM glucose 6-phosphate followed by 5 mM mannose 6-phosphate (M6P) in the column buffer.

SDS-PAGE and Western blot analysis

Samples were analyzed by SDS-PAGE under non-reducing and reducing conditions as described (Laemmli et al., 1970). Gels were stained using the silver staining method (Blum H et al., 1987). For immunological detection of MPR proteins, aliquots of the purified proteins were electrophoresed, and the proteins transferred to nitrocellulose membrane as described (Towbin H et al., 1979).For the detection of MPR 300, 10 µg of affinity purified goat MPR 300 IgG was used as the primary antibody, purified goat MPR 300, *unio* MPR 300 (invertebrate, mollusc) and goat MPR 46 were used as positive controls. For the detection of MPR 46, antiserum raised against purified goat MPR 46 protein, (1 : 500 dilution) as well as 10 µg of the MSC1 antibody (affinity purified antibody obtained from the MSC1 antiserum raised against the cytoplasmic tail of the human MPR 46 protein) were used. For analysis of cathepsin D, 50 µg of total cellular protein was subjected to immunoblotting and the blot was probed with antiserum specific for cathepsin D. The blots were probed using the enhanced chemiluminescence (ECL) light-based immuno-detection system (Amersham Pharmacia Biosciences).

Immunofluorescence

Immunostaining analysis was performed as described (Praveen Kumar et al., 2007), using a confocal laser scanning microscope. Primary antibodies were as described

above; fluorescence-conjugated secondary antibodies were used are goat anti-rabbit-IgG-FITC and goat anti-mouse-IgG-cy5 (Chemicon, India) respectively.

Metabolic labeling and immunoprecipitation

MEF cells were incubated in methionine-free medium for 1 h and then labeled with ^{35}S-methionine in the same medium containing 5% dialyzed FCS. During the chase (8hr), the medium was supplemented with 0.25 mg/ml l-methionine Immunoprecipitation for newly synthesized Arysulfatase A from cells and media was carried out as described previously (Waheed et al., 1982) with rabbit antibodies against arylsulfatse A, quantification of band intensities was done with densitometer.

Co-immunoprecipitation

Co-immunopreciptation for β-hexosaminidase and expressed starfish MPR 46 was done as described (Praveen Kumar et al., 2007).

Chromatography on MPR 46 and MPR 300 Affinity gel

MPR 46 and MPR 300 were purified from goat liver as described and coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer's manual secreted media obtained after labelling with ^{35}S-methionine were subjected to MPR affinity chromatography as described (Braulke et al., 1990). The mannose 6-phosphate eluates were precipitated with 10% trichloroacetic acid; pooled and dissolved in 50 µl of 0.4 M Tris-HCl, heated for 5 min at 95 °C in 10 mM dithiothreitol, 1% SDS; and analyzed by SDS-PAGE (10%) and fluorography.

RESULTS

Molecular cloning and sequencing of starfish MPR 46

We obtained a full length cDNA clone for putative starfish MPR 46 protein. Total RNA isolated from starfish gonads (Fig.4.7A) was used for synthesis of cDNA by RT-PCR with oligo (dT) as primer. In a PCR reaction a 819bp fragment was amplified by employing the combinations of the degenerative conserved sequence primers shown under methods. The sense and antisense, primers given in bold resulted in amplification of the fragment (Fig.4.7B, Lane1), the amplified fragment was cloned into TA vector (pTZ57R) and transformed into DH5α *E.coli* cells. Cloned plasmid DNA was isolated, digested with EcoRI and Hind III, resulting in release of 819 bp insert (Fig.4.7C lane 5). In northern blot analysis the transcript size of the starfish MPR 46 gene was found to be ~2.2kb (Fig.4.7D). The amino acid sequence deduced from the starfish MPR 46 cDNA (Fig. 4.8) clone was aligned with other known vertebrate sequences by multiple sequence alignment. Fig. 4.9 shows the numbering of the sequence obtained for starfish protein. The protein consists of a short signal peptide (1-20 amino acids), a N-terminal region (21-182 amino acids), a trans membrane domain (183-202 amino acids) and a cytoplasmic tail (203-273 amino acids). The extent of similarity of the starfish protein to other known vertebrates varied differently. It exhibited 46% similarity with human, 50% with the bovine, 54% with the mouse, 48% with the goat, 60% with the chicken, 54% with the *xenopus,* 25% with the killifish and 52% with the zebra fish sequences. The cysteine residues responsible for the proper folding of the protein are conserved from starfish to mammals. It is also known that amino acids such as the glutamine, histidine, arginine and tyrosine (indicated as $ in Fig. 4.9) in the extra cytoplasmic domain are important for the mannose 6-phosphate binding in the vertebrates. Interestingly

these residues are also conserved in the starfish protein sequence (Gln 88, His 124, Arg 130, and Tyr 162). Additionally an aspartic acid residue is also found in the starfish sequence at position 120 that may be involved in the metal binding. Furthermore, from our earlier studies it is clear that the chicken, fish and mollusc MPR 46 proteins bind phosphomannan gel in the absence of salt and in the presence of three divalent metal ions (Siva Kumar et al., 1999;2002), although it is still not clear why the MPR 46 protein in these species binds under these conditions only. The starfish protein exhibits similar property as the chicken, fish and mollusc receptors. The transmembrane domain as well as the cytoplasmic tail of the starfish protein is also highly conserved. In particular the endocytosis signals (*YRGY* amino acids 251-254) the acidic clusture dileucine motif (DxxLL, 267-272) which are known to interact with the GGAs (Golgi associated gamma adaptin ear containing ARF binding proteins) for recycling are highly conserved in the starfish sequence also.

Expression and functional characterization of starfish MPR 46

The starfish MPR 46 cDNA was cloned into the pcDNA6V5/His expression vector to make a biochemical and functional characterization of the receptor. Stable transfections were made in MPR-deficient mouse embryonic cells (mpr (-/-) MEF) that missort up to 98% of soluble lysosomal enzymes to the medium due to the lack of sorting MPRs. In these cells, the missorting of lysosomal enzymes, causing accumulation of non-degradated material (inclusions), can be rescued by the expression of a starfish receptor protein that re-establishes functional sorting. The sub cellular distribution of starfish MPR 46 in the transfected cells was studied by immunocytochemical analysis. The results revealed an intracellular distribution of the starfish MPR 46 protein as detected by MSC1 antibody (Fig.4.10F). The expressed

starfish MPR 46 was localized in prelysosomal structures and colocalized with the lysosomal marker protein Lamp1 (Fig.4.10G).

Biochemical properties of expressed starfish MPR 46

To analyze whether starfish MPR 46 binds mannose-6- phosphate residues, we prepared membrane fractions from starfish MPR 46-expressing mpr (−/−) MEF cells as described under materials and methods. The membrane extracts were passed over a multimeric mannose-6-phosphate phosphomannan ligand matrix. Unbound fraction, wash glucose 6-phosphate and mannose 6-phosphate elutions were subjected to SDS-PAGE analysis and the proteins detected by silver staining. From (Fig. 4.11A), it is evident that the expressed protein could be specifically eluted from the PM gel using mannose 6-phosphate. In addition to the monomeric receptor band, the dimeric protein band was also detectable. The MPR 46 displays similar pattern in the goat, zebra fish (Praveen Kumar et al., 2007; Suresh et al., 2005). Furthermore by western blot analysis it has also been shown that the expressed starfish protein can be recognized by the goat MPR 46 antiserum, MSC1 antibody and 218 peptide antiserum [Fig.4.11B, C and D (goat 46, MSC1 and 218 antiserum)]. With the 218 antiserum, both the monomeric and the dimeric bands could be seen.

Sorting of phosphorylated ligands

The efficiency of sorting man 6-phosphorylated ligands by the expressed starfish protein was analyzed, using the secretions obtained from the ^{35}S methionine labeled mock transfected and transfected cells. These were separately passed through affi-gel 10 that contains immobilized purified goat MPR 46 and 300 proteins. After extensive washing, bound ligands were eluted with 5mm Man- 6-P and characterized by SDS-PAGE and fluorography. From Fig 4.12A, it is evident the mock transfected

cells there are a large number of bands visible in the secretions, while in the transfected cell secretions there are only a few bands, suggesting that the transfected protein is able to retain the phosphorylated ligands.

Sorting of newly synthesized arylsulfatse A and cathepsin D in MPR-deficient fibroblasts expressing starfish MPR 46

To determine the sorting of newly synthesized lysosomal enzymes, fibroblasts were metabolically labeled in the with [^{35}S] methionine as described under methods. From the secretions and the soluble cell extracts of the mock transfected and transfected cells, arylsulfatse A was immunoprecipitated. In mock transfected cells, Arylsulflatase A was secreted out while in transfected cells, 10.7% of the labeled enzyme was recovered in the secretions and 89.3% was sorted to lysosomes efficiently in intracellularlly (Fig 4.12B). This clearly demonstrates that the bulk of newly synthesized Arylsulfatse A is sorted to lysosomes in starfish MPR 46 expressing fibroblasts. Furthermore, in the transfected cells, in parallel to arylsulfatse A, sorting of cathepsin D and its colocalization with LAMP1 protein (a lysosomal membrane marker protein) was also demonstrated by immunofluorescence using cathepsin D antiserum. Compared to the mock transfected cells (Fig 4.13, upper panel), in the transfected cells (Fig.4.13, lower panel).

Cathepsin D in lysosomes of starfish MPR 46 expressing fibroblasts

The steady-state concentration of cathepsin D varied between 52.9% and 93.6% in mock transfected and transfected cells as analyzed by western blotting (Fig.4.14A). In starfish MPR 46 expressing fibroblasts, the precursor, Intermediate forms and 30-kDa mature forms of cathepsin D are retained in inracellurly with the bulk amount (93.6%) and in secretions only the intermediate forms appears with low concentration (6.4%), wherein, mock transfected cells the precursor form and

intermediate forms are detectable in intracellular as well as in secretions almost with similar concentrations and the mature form is barely detectable. The specific interaction was further confirmed by co-immunoprecipitation experiment with beta-Hexosaminidase antiserum. Preimmune serum was used as a control (Fig. 4.14B lane 1). Man 6-P dependent binding was also analyzed in presence and absence of Man 6-P (Fig. 4.14B lane 2). The specific identification of the goat MPR 46 monomeric and dimeric forms indicates the mannose 6-phosphate dependent binding of starfish MPR 46 protein with the hexosaminidase (Fig.4.14B lane 3).

Figure 4.6: Schematic representation of cloning and expression of starfish MPR 46 into pcDNA6V5/His-Tag Vector.

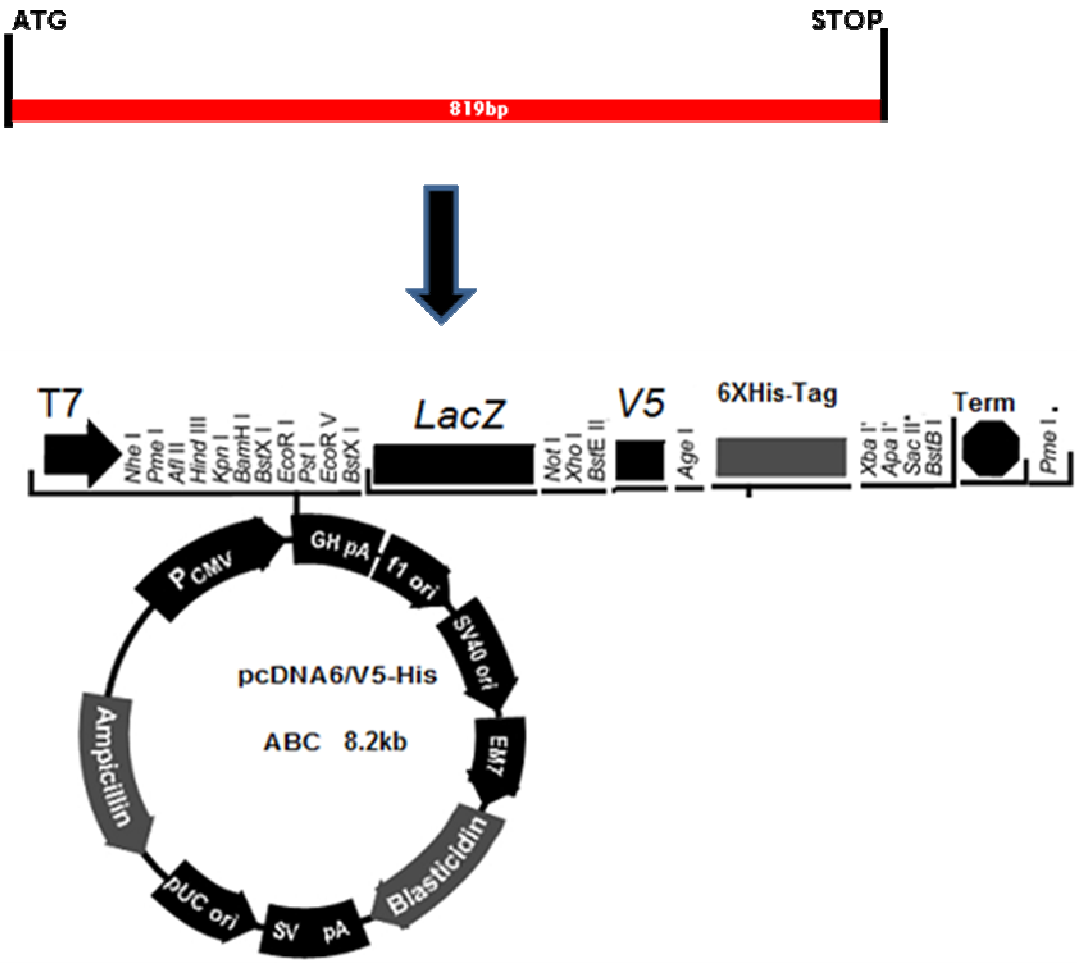

ATG
STOP
819bp
T7
LacZ
V5
6XHis-Tag
Term
Nhe I
Pme I
Afl II
Hind III
Kpn I
BamH I
BstX I
EcoR I
Psi I
EcoR V
BstX I
Not I
Xho I
BstE II
Age I
Xba I
Apa I
Sac II
BstB I
Pme I
P CMV
GH pA
f1 ori
SV40 ori
EM7
Blasticidin
SV pA
pUC ori
Ampicillin
pcDNA6/V5-His
ABC 8.2kb

Figure 4.6

Figure 4.7: Molecular cloning of starfish MPR 46

A). Total RNA isolated from starfish gonad tissues.

B). PCR amplification of full length starfish MPR 46. Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, standard DNA ladder, Lane 2, amplified product (819 bp).

(C).Restriction digestion analysis. Lane 1 DNA ladder (100bp-10kb), lane 2 Vector alone, lane 3 and 4 single digested with (EcoRI) and Hind III respectively, Lane 5 Vector digested with EcoRI and Hind III [(←) fragment released from the vector after digestion with EcoRI and HindIII].

D).Northern Blot Analysis. 15 µg of total RNA isolated from goat liver tissue and subjected to denaturing 1% agarose gel electrophoresis, transferred to hybond-N-nylon membrane and hybridized with ^{32}P labeled starfish MPR 46 full length cDNA clone (819 bp).

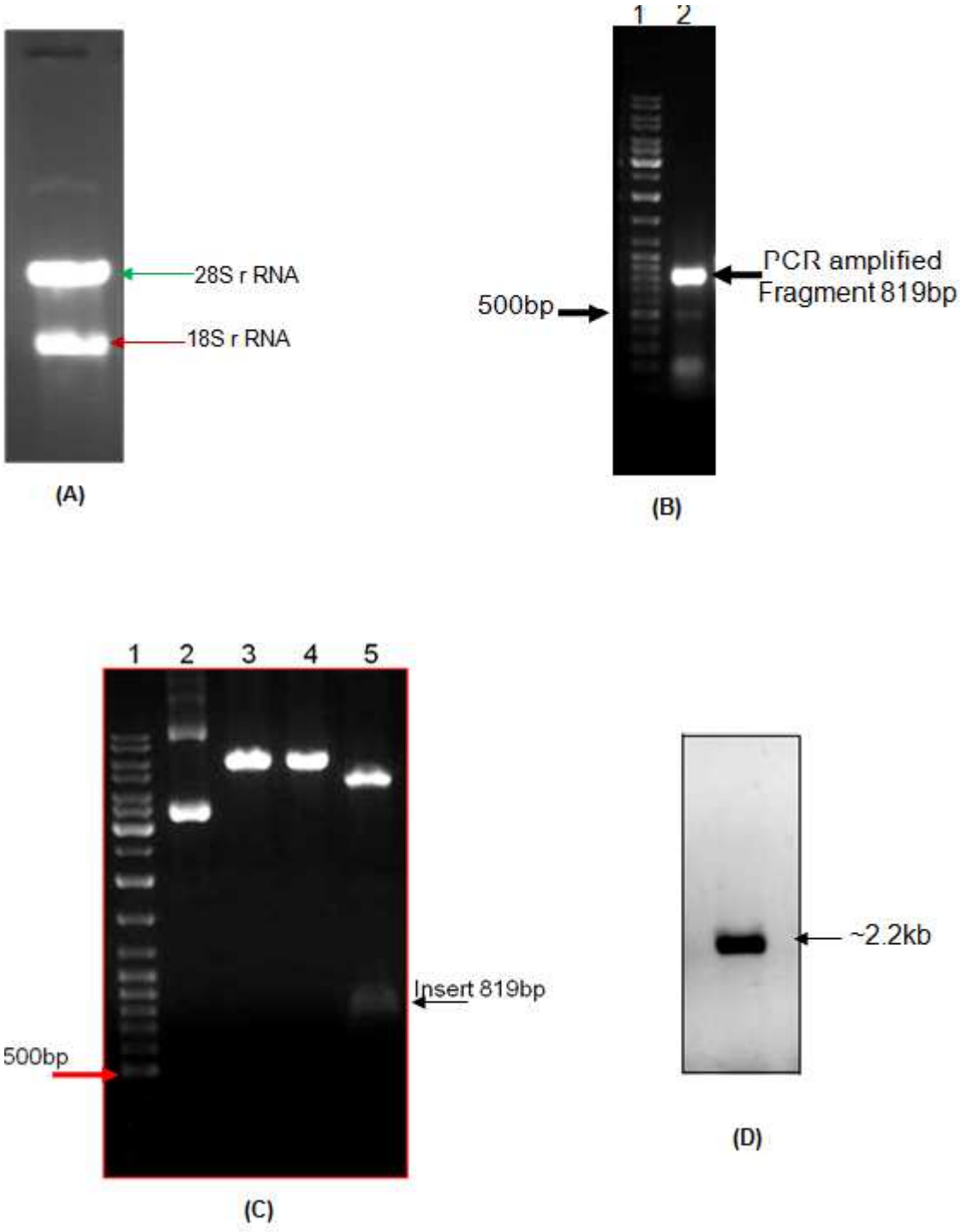

28S r RNA
18S r RNA
(A)
1 2
500bp
PCR amplified
Fragment 819bp
(B)
1 2 3 4 5
Insert 819bp
500bp
(C)
~2.2kb
(D)

Figure 4.7

Figure 4.8: Nucleotide and deduced amino acid sequence of the starfish MPR 46 protein.

cDNA sequence

```
ATGCTGAACAGTGTAAGGGGTATTACTCCCCTATTTACTTTCGCCGGAGCAGTG
GAGGCCAGGTTCACGTGGGCTGACCGCCGAGAGAGCGCCTGTGATGTGATGAGA
GTACTGAGTCCCAATGGAAAAGCCCTGAAGGAACCCCTGAGCCAAATAAGATTT
ACCAAGGCTCTGAAGACTAGCAGTGGCGACTGTTCCGACCTCGTACACGTGTGG
TGCCGAGCCATAAATGCCTTTTTGCATGATTTTGCTGGCCTAGTACAAATGGAT
AGGAAGAAGACTACAAATCTGGGAAGAATCAATGAAACCCAGGTCATCGATGGC
GGTGAAAGGATGCTGCTCGTAATCGAAGAGGGTGATAAGGATAGGCACTGCAGT
AGTGAGGGTAGAAAGGCTCTGCTAGTGATTTCTTGCAAGCGAGGATTTACAGCG
GGTCCACCCACTCTGATTTCAGAAGAGAGGAAAAAGGAGCACGACTGTTTCTAC
CTCTTTGAGATGGACACTAATGTGGCTTGTCCAGCTGAGTCCCACCTCAGCACT
GGCTCCATTCTACTAATCACGTTTTCTGCACTAGTTACAGTCTATTTGATAGGC
GGCTTTCTATATCAACGACTATCGTGGAGCATAAGCATGGAGCAGTTCTCTCAC
TTTGCCAAGACTTGGGCAATTCGGCAAGACATCGGCAATTTGACAGCGGATGGC
TGTGACTTTGTCTGCCGATCTCGAAATCGAGAGGAACCGGTACCAACATATCGT
GGTGTGGGTACAGACCAGTTGGGTGATGAGCCGGAGGAACGGGATGACCACTTG
CTACCGATG
```

Deduced Amino acid sequence

```
MLNSVRGITPLFTFAGAVEARFTWADRRESACDVMRVLSPNGKALKEPLS
QIRFTKALKTSSGDCSDLVHVWCRAINAFLHDFAGLVQMDRKKTTNLGRI
NETQVIDGGERMLLVIEEGDKDRHCSSEGRKALLVISCKRGFTAGPPTLI
SEERKKEHDCFYLFEMDTNVACPAESHLSTGSILLITFSALVTVYLIGGF
LYQRLSWSISMEQFSHFAKTWAIRQDIGNLTADGCDFVCRSRNREEPVPT
YRGVGTDQLGDEPEERDDHLLPM
```

Figure 4.8

Figure 4.9: Multiple sequence alignment of amino acids of MPR 46 protein from different species. ( ) indicates the signal peptide cleavage site predicted by the signal 3.0 server (http://www.cbs.dtu.dk/services/SignalP/); ($) indicates the conserved M6P ligand binding residues ;(#) indicates predicted N-glycosilation sites predicted by the NetNGlyc 1.0 server; 1,2,3 indicate the conserved cysteine residues; the underline sequence represents the trans membrane domain; bold italized characters mark the tyrosine dependent endocytosis motif *YRGV*;Boxed sequence indicate the dileucine motif interacting with sorting GGAs;(*) marks identical amino acid residues;(:) related amino acid residues;(.) predominantly the same amino acid residue.

```
CLUSTAL W (1.8) multiple sequence alignment

                    1                                                    46
BOVINE              MMSPLHSSWRTGL-----LLLLLFSVAVRESWQ▼TE-EKTCDLVGEKGKESEKELALLKRL
GOAT                -MSPLHSSWRTGL-----LLLLLFSMAVRESWQ▼TE-EKTCDLVGEKGKESEKELALLKRL
HUMAN               -MFPFYSCWRTG------LLLLLLAVAVRESWQ▼TE-EKTCDLVGEKGKESEKELALVKRL
MOUSE               -MFPFSGCWRTEL-----LLLLLLAVAVRESWQ▼E-EKSCDLVGEKDKESKNEVALLERL
CHICKEN             ----MSSHCHTSA-----VLVVFMALAAGVGA▼EPLSEKSCDVVGDESTESQMEKALLKKL
XENOPUS             ---MCAPLWCVS-------IAALLAIAAG▼ENLVDS----CQLVGG-DRKSVTEQQLLAKL
STARFISH            ---MLNSVRGITP-----LFTFAGAVEA▼RFTWADRRESACDVMR---VLSPNGKALK---
KILLIFISH           --MKLFRIARRGYVLLWALLAQLVLC▼RSGVFAGDG-TKACVLAH----ETVSERKVLDRL
ZEBRAFISH           ---MLLSVR---------IITPLFVLFAGVQA▼RFN-TSNCKLVS----DSESQRKALRLL
                                      :                  * :       :
                   47                          1                $                100
BOVINE              TPLFNKSFESTVGQSP-DMYSYVFRVCREAGNHSSG--AGLVQIN-KSNGKETV--VGRF
GOAT                TPLFNKSFESTVGQSP-DMYSYVFRVCREAGNHSSG--AGLVQIN-KSNGKETV--VGRF
HUMAN               KPLFNKSFESTVGQGS-DTYIYIFRVCREAGNHTSG--AGLVQIN-KSNGKETV--VGRL
MOUSE               RPLFNKSFESTVGQGS-DTYSYIFRVCREASNHSSG--AGLVQIN-KSNDKETV--VGRI
CHICKEN             EPLSQIRFNTTVEIGTTENYAYHFRVCREVNSSLHDF-AGLVQMD-RQSGKTTV--IGRI
XENOPUS             APLKGKRFEAKTQEGS-DIYTYTFVVCGRVNNDSKSTNEGLVQSK-DGSKDTSV--IGRI
STARFISH            EPLSQIRFTKALKTSSGDCSDLVHVWCRAINAFLHD-FAGLVQMD-R--KKTTN--LGRI
KILLIFISH           EPLAFKNLTVESKNEK-DSYTYVFQLCGDAGG-VPG--AGVIQKNMKTPEKPIVTNIGSY
ZEBRAFISH           EPLTNQNFTTEGQ-EK-EKYSYIFQVCGDAGG-VKN--AGLIQQ--EKGGKTIR--IGDY
                     **    :         :     .  *   .    .   *::*       .     :*
                   101                        $2    $                   $ $    159
BOVINE              NETQIFNGSNWIMLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQD
GOAT                NETQIFNGSNW-MLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQD
HUMAN               NETHIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLADNFNPVSEERGKVQD
MOUSE               NETHIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLAANFNPVSEERGKVQD
CHICKEN             NETQVFNGSDWIMLIYKGGDSYGRHCSGEKRRAVIMISCKRGITASSFSIISEEREKEQD
XENOPUS             NDTHIINGSDWIMLYYRSGDKYDTHCNNEARKAMVMISCNKGTVGDGFTVIQEERNKSSE
STARFISH            NETQVIDGGERMLLVIEEGDK-DRHCSSEGRKALLVISCKRGFTAGPPTLISEERKKEHD
KILLIFISH           KATKAIGGSDWVMLIYEQGDKYDSHCQKEARKAIIMISCSKNTDVGALEVILEENERDTD
ZEBRAFISH           SKTVATAGSDWVLLIYEGGEKYDSHCSSEERKAMIMISCSSSSKS-AFSVVMEENQKQKN
                     . *    *.:  :*  . *:. . **  * *:*:::***.         : **. :  :
                   160 $        3                                              217
BOVINE              CFYLFEMDSSLACSPEISHLSVGS**ILLVTLASLVAVYIIGGFLY**QRLVVGAKGMEQFPHL
GOAT                CFYLFEMDSSLACSPEISHLSVGS**ILLVTFASLVAVYIIGGFLY**QRLVVGAKGMEQFPHL
HUMAN               CFYLFEMDSSLACSPEISHLSVGS**ILLVTFASLVAVYVVGGFLY**QRLVVGAKGMEQFPHL
MOUSE               CFYLFEMDSSLACSPEVSHLSVGS**ILLVIFASLVAVYIIGGFLY**QRLVVGAKGMEQFPHL
CHICKEN             CFYLFEMDSSVACPAEDSHLSTGS**ILLITFSALVTVYIVGGFLY**QRLIVGAKGMEQFPHF
XENOPUS             CFYLFEMDSSLACPPEESHLSAGS**ILLIVFAVLVAVYLIGGFLY**QRFVVGAKGMEQFPNI
STARFISH            CFYLFEMDTNVACPAE-SHLSTGS**ILLITFSALVTVYLIGGFLY**QRLSWSIS-MEQFSHF
KILLIFISH           CFYLFELDSSAVCPPVESRLSAGS**IILIIIFCLVAVYLIGGFLY**QRLIV-----------
ZEBRAFISH           CYYLFELDTTAVCPAVSSKLSAGS**IVLIVVISSLTVYIIGGFLY**QRLVVGAKGVEQFPNF
                     *:****:*:. .*..  *:**.***:*: .   ::**::*******:
                   218                                                        273
BOVINE              A----FWQDLGNLVADGCDFVCRSKPRNVPA-A***YRGV***GDDQLGEESEERDDHLLPM
GOAT                A----FWQDLGNLVADGCDFVCRSKPRNVPA-A***YRGV***GDDQLGEESEERDDHLLPM
HUMAN               A----FWQDLGNLVADGCDFVCRSKPRNVPA-A***YRGV***GDDQLGEESEERDDHLLPM
MOUSE               A----FWQDLGNLVADGCDFVCRSKPRNVPA-A***YRGV***GDDQLGEESEERDDHLLPM
CHICKEN             A----FWQDLGNLVADGCDFVCRSKPRNVPA-A***YRGV***GDDQLGDESEERDDHLLPM
XENOPUS             T----LWQELGNLSADGCDFVCRSRPRTSET-A***YRGV***GEDQLGEEPEERDDHLLPM
STARFISH            AKTWAFWQDIGNLTADGCDFVCRSRNREEPVPT***YRGV***GTDQLGDEPEERDDHLLPM
KILLIFISH           ----------------------------------------------------------
ZEBRAFISH           A----FWSEIGNLSADGCDFVCRSRGNREEPPTYRGVGTEPLGEEPEERDDHLLPM
```

Figure 4.9

Figure 4.10: Sub-cellular Localization of MPR 46 protein. (A) & (E) incubation with Lamp-1 followed by FITC (green) conjugated secondary antibody, of mpr $^{(-/-)}$ MEF cells expressing starfish MPR 46 and mock transfected mpr $^{(-/-)}$ MEF cells respectively. **(B) & (F)** incubation with MSC1 antibody followed by Cy3 (red) conjugated secondary antibody, of mock transfected mpr $^{(-/-)}$ MEF cells and starfish MPR 46 expressing cells respectively. **(C)** merged image of **A** and **B**, **(G)** merged image of **E** and **F**. **D** and **H** are the corresponding transmission images.

Mock transfected mpr$^{(-/-)}$ MEF cells

Starfish MPR 46 expressing mpr$^{(-/-)}$ MEF cells

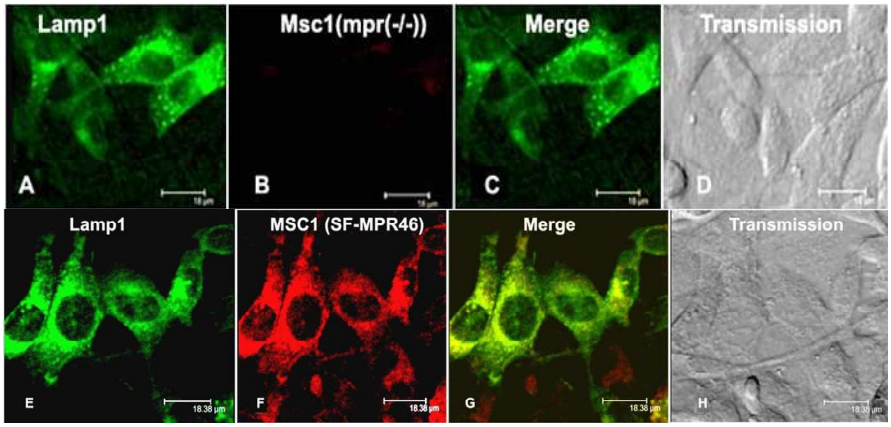


Figure 4.10

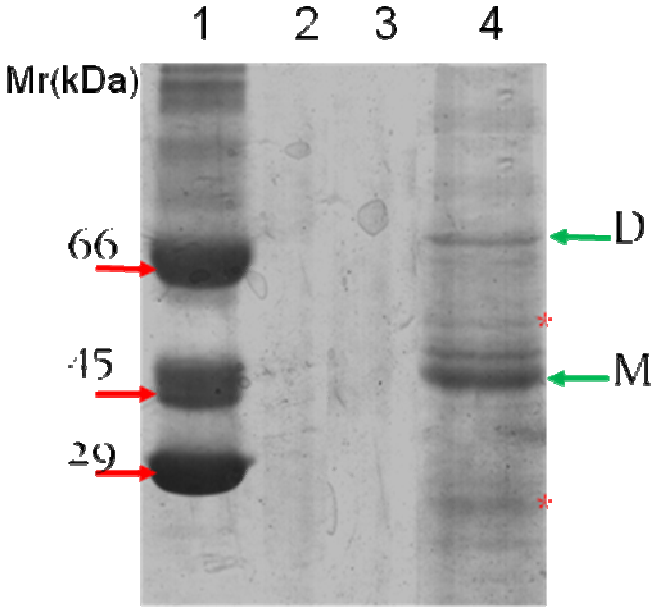
Figure 4.11 Biochemical characterization of expressed starfish MPR 46.

(A) Purification of expressed starfish MPR 46 protein on PM gel. Proteins were separated on 10% SDS-PAGE and silver stained; Lane 1 Molecular weight markers, lane 2 wash, lane 3 Glucose 6-phosphate eluate, lane 4 mannose 6-phosphate eluate showing monomeric (M) and dimeric (D) forms.

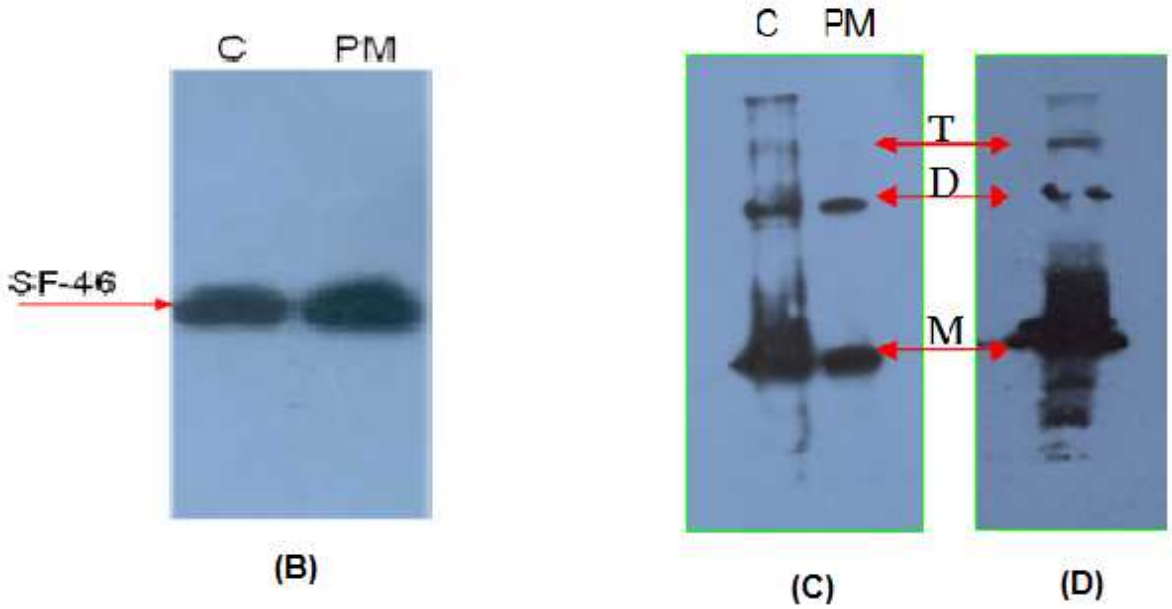
B) Western blot analysis of the expressed protein, Lane 1, Expressed protein purified on phosphomannan gel (**PM)** , lane 2, **(C)** Cell lysate from the cells expressing starfish MPR 46 protein, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with Goat MPR 46 antiserum.

C) Western blot analysis of the expressed protein, Lane 1, Expressed protein purified on phosphomannan gel (**PM)** , lane 2, **(C)** Cell lysate from the cells expressing starfish MPR 46 protein, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with MSC1 antibody antiserum.

D) Western blot analysis of the expressed protein and 218 peptide antibody. Blots were developed with ECL reagent; both the monomeric (M) and dimeric (D) forms were observed in cell lysate as well as PM elution (B&D).



(A)

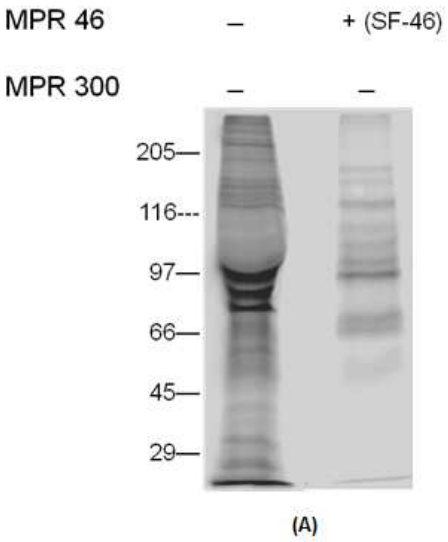


(B) (C) (D)

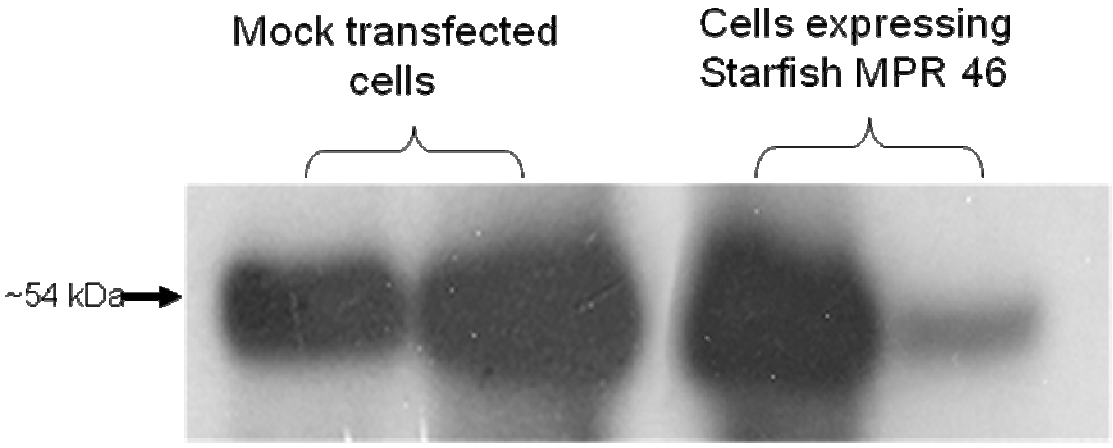
Figure 4.11

Figure 4.12: (A) Secretion of M6P-containing polypeptides in mpr (-/-) MEF fibroblasts expressing starfish MPR 46.Cells expressing SFMPR46 was tested for their ability to retain the missoting of lysosomal enzymes; cells were pulsed with [^{35}S] methionine chased. The labelled phosphorylated ligands secreted in the culture medium were purified on MPR affinity columns and characterized by SDS-PAGE and fluorography. Lane 1 is secretions from mock transfected cells, lane 2 starfish MPR 46 transfected cells.

(B) Biosynthesis of Arylsulfatase A in mock transfected and mpr $^{(-/)}$ MEF fibroblasts expressing starfish MPR 46. Cultured fibroblasts were metabolically labeled with [^{35}S] methionine. Arylsulfatse A was immunoprecipitated from equal amounts of trichloroacetic acid-insoluble radioactivity of cells **(C)** and corresponding aliquots of the secreted culture media **(M)** and subjected to SDS-PAGE and fluorography. The relative level of newly synthesized Arylsulfatse A and the percentage of newly synthesized protein detectable in the secretions were calculated by densitometry analysis and are given at the bottom.



(A)



(B)

Figure 4.12

Figure 4.13: Intracellular retention and sorting of cathepsin D. **(A) & (E)** incubation with Lamp-1 followed by FITC (green) conjugated secondary antibody, of mock transfected and mpr$^{(-/-)}$ MEF cells expressing starfish MPR 46 cells respectively. **(B) & (F)** incubation with cathepsin D antiserum followed by Cy3 (red) conjugated secondary antibody, of mock transfected mpr $^{(-/-)}$ MEF cells and starfish MPR 46 expressing cells respectively. **(C)** Merged image of **A** and **B, (G)** merged image of **E** and **F,** clearly shows the extensive co-localization of Lamp-1 and cathepsin D, thus supporting the function of starfish MPR 46 protein to bind and target cathepsin D to lysosomes. **D** and **H** are the corresponding transmission images.

Mock transfected mpr$^{(-/-)}$ MEF cells

Starfish MPR 46 expressing mpr$^{(-/-)}$ MEF cells

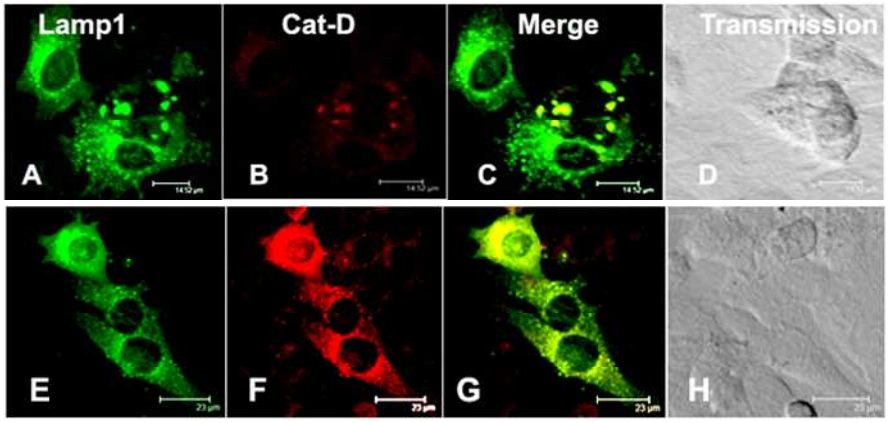


Figure 4.13

Figure 4.14 Quantification of cathepsin D level.

A) Quantification of cathepsin D protein presents in the cell lysate (C) and the 12 hr culture medium (M) from the mock transfected mpr $^{(-/-)}$ MEF cells. Quantification of cathepsin D protein present in starfish MPR 46 protein expressing mpr$^{(-/-)}$ MEF cell lysate (C) and the 12 hr culture medium (M).

B) Co-immunoprecipitation of the cell lysates prepared from mpr (−/−) MEF cells transfected with starfish MPR 46 using hexosaminidase antiserum. Lane 1, cell lysate with preimmune serum, lane 2, cell lysate in presence of 5 mM M6P, lane 3, cell lysate in the absence of 5 mM M6P detected by MSC1 antibody. **M** and **D** represent the dimeric and monomeric forms of the starfish MPR 46 receptor protein.

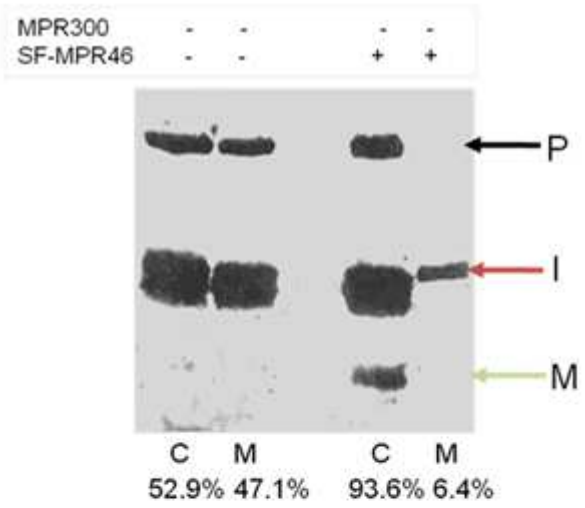


Figure 4.14

DISCUSSION

The biogenesis of lysosomes requires the correct sorting of >50 acid hydrolases in cells from their site of synthesis in the endoplasmic reticulum to their final destination the lysosomes. The 46kDa cation-dependent mannose 6- phosphate receptor (CD-MPR) and the 300kDa cation-independent MPR (CIMPR) divert these soluble enzymes from the secretory pathway by delivering their cargo bearing Man-6-P on N-glycans from the *trans* Golgi Network (TGN) to endosomes. Mammalian mannose 6-phosphate receptors (MPR 46 and 300) are homologous proteins with distinct functions which have been well characterized. Only MPR 300 protein has been shown to be a multifunctional protein (Dahms and Hancock, 2002). From the last few years we have been working on a comparative analysis of the MPRs in non-mammalian and in the invertebrate species to establish their phylogenetic origin, evolutionary history and functional significance in the animal kingdom. We were so far successful in identifying, purifying and characterizing these proteins from fish to mammals and established that they are evolutionarily conserved (Suresh et al., 2005). Although both putative receptors have been identified and purified from the invertebrates (echinodermates and molluscs), their detailed functional characterization is underway in our laboratory. In the present study the starfish MPR 46 has been cloned, expressed in mouse embryonic fibroblast cells lacking both receptors, and characterized.

Although the MPR clearly has a major role in lysosomal enzyme sorting in the vertebrate cells, its role in the invertebrates is not understood. In lower eukaryotes, such as *Saccharomyces, Trypanosoma,* and *Dictyostelium*, lysosomal enzymes are targeted without the aid of identifiable MPRs. The slime mold *Dictyostelium discoideum* produces a novel methyl-phosphomannose sequence on some of its

lysosomal enzymes that can be recognized *in vitro* by the mammalian CI-MPR (not the CD-MPR). However, despite the presence of a GlcNAc-P-T that recognizes α-1–2 linked mannose residues, no receptor for the phosphorylated mannose residues has been found in these organisms. In contrast to this situation, the protozoan *Acanthamoeba* produces a GlcNAc-P-T that does show specific recognition of lysosomal enzymes (Gabel et al., 1984; Couso et al., 1986). Since we have already established that the vertebrate receptors are highly conserved proteins, and identified the putative receptors in the molluscs, it became necessary to extensively characterize the invertebrate receptors (echinodermates and molluscs). Sequence by cloning and functional characterization of the invertebrate receptors (echinodermnates and molluscs) should conclusively establish the functional significance and phylogenetic origin of the two receptors, and also about the physiological role of these two receptors in normal and pathological conditions. As a first contribution to gain new insights into the invertebrate receptors, in the present study, we set out first to purify the receptors from the highly evolved invertebrates, echinodermates that fall above the molluscs in the evolutionary tree. We further cloned the gene for the starfish MPR 46 protein and studied its function by expressing it in MPR-deficient mouse embryonic cells (mpr (-/-) MEF). The phosphomannan binding ability of the expressed cDNA and the transcript size of ~ 2.2 kb obtained by northern analysis suggest that the expressed cDNA represents the MPR 46 protein. The amino acid residues critical for ligand binding by the MPR proteins have been identified (Sun et al., 2005). Glutamine, histidine, arginine and tyrosine are the four important amino acids that have been identified and characterized to be important in ligand binding in mammalian MPR 46 proteins and these are also conserved in the starfish protein. Additionally from the crystal

structure of bovine MPR 46 protein, it is also known that two cysteine residues, histidine, arginine, aspartic acid and glutamic acid are important for interaction with Mn^{+2} (Suresh et al., 2004; Sun et al., 2005). We found that in starfish sequence, these residues are also highly conserved like in other vertebrate species and the aspartic acid at position 120 might play a crucial role in metal binding.

A key function of MPRs is to segregate newly synthesized acid hydrolases from secretory proteins at the TGN. In both MPRs this sorting signal is composed of a cluster of acidic residues followed by a dileucine motif at the carboxy terminus of the cytoplasmic domain [referred to as an acidic cluster–dileucine motif (DxxLL)] and also involves several binding sites for AP-1 (Ghosh et al.,2003). Carboxy-terminal DxxLL motifs and conserved AP-1, GGAs binding sites are present in the cytoplasmic tails of starfish MPR46. Following delivery of acid hydrolases, mammalian MPRs do not enter lysosomes and are instead recycled from late endosomes to the TGN in processes mediated by the protein TIP47 (Ghosh et al.,2003). TIP47 recognizes a phenylalanine–tryptophan motif in the cytoplasmic tail of the bovine CD-MPR, and this motif is present in the starfish MPR46 also. Heterologous expression of the starfish MPR 46 in mammalian cells revealed an intracellular distribution (Fig.10F) similar to the goat MPR 46 (Praveen Kumar et al., 2007). This distribution fits very well to the demonstrated lysosomal sorting function of the starfish receptor. The common distribution and the common sorting function both provide evidence that starfish MPR 46 protein functions like its mammalian counterpart, recognized by key organizers responsible for this specific vesicular sorting pathway. These data provide strong evidence to suggest that in the model organism, starfish (invertebrate) the putative lysosomal sorting receptors behave like the mammalian proteins, giving new insights towards the understanding of the

importance of lysosomal enzyme sorting process in the invertebrates. This was further supported by additional experiments where we found that an antiserum to the mollusc α-fucosidase enzyme showed immunological cross-reactivity with a protein from the soluble extracts of the starfish (both from the total animal extract as well as from the gonads) suggesting that the starfish may have a related enzyme (data not shown).

In summary, the results of our study clearly demonstrate that i) the highly evolved invertebrates, starfish contain the putative MPR proteins (MPR 46 and 300), that can be affinity purified on the multivalent phosphomannan gels, like the mammalian and non-mammalian vertebrate receptors, ii) the biochemical and immunological properties of the starfish receptors resemble the mollusc, mammalian and other receptors characterized, iii) the star fish MPR 46 exhibits high degree of structural homology to other vertebrate receptors, iv) the starfish also contains a glycosylated α-fucosidase enzyme that appears to be cross-reacting with the mollusc enzyme antiserum,(suggesting antigenic similarities among these invertebrate enzymes) and v) Starfish MPR 46 expressed in Immortalized mouse embryonic fibroblasts cells (mpr- /- MEF) which are deficient in MPR 46 and MPR 300 shown a clear evidence for the functional significance of starfish MPR 46 protein. This is the first report on the functional characterization of MPR 46 from the highly evolved invertebrate starfish. It is unknown whether the mannose-6-phosphate-dependent sorting system of lysosomal enzyme recognition is established identically in invertebrates and mammals. This has to be addressed by an extensive characterization of the components of the lysosomal system in starfish, i.e. by cloning and characterization of lysosomal enzymes, the sorting GGAs and the second MPR, the MPR 300. The new data obtained on the starfish protein has now laid the foundation to clone the

genes for the mollusc receptors and to establish the structure-functional significance of the receptors in the invertebrate species.

A manuscript describing the characterization of the starfish receptors is under review with Glycoconjugate Journal.

Sivaramakrishna Yadavalli and Siva Kumar Nadimpalli # (2008) Mannose 6-phosphate receptors (MPR 300 and 46) from the highly evolved Invertebrate *Asterias rubens* (Echinodermate): Biochemical and functional characterization of MPR 46 protein. ***Glycoconjugate Journal – Revised version submitted.***

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