Functional characterization of mannose 6-phosphate receptors (MPR 46) from goat and chicken: MPR 300 dependent targeting of lysosomal enzymes in molluscs

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

Praveen Kumar Amancha

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046
INDIA.

Enrollment No: 04LBPH03 **October 2008**

University of Hyderabad

School of Life Sciences,

Department of Biochemistry

CERTIFICATE

This is to certify that this thesis entitled "**Functional characterization of mannose 6-phosphate receptors (MPR 46) from goat and chicken: MPR 300 dependent targeting of lysosomal enzymes in molluscs"** submitted to the University of Hyderabad by **Mr. Praveen Kumar Amancha** 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

School of Life Sciences.

University of Hyderabad

School of Life Sciences,

Department of Biochemistry

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.

A. Praveen Kumar

Date:

Prof. N. Siva Kumar
(*Supervisor*)

University of Hyderabad

School of Life Sciences,

Department of Biochemistry

ACKNOWLEDGEMENTS

*It is difficult to overstate my gratitude to my Ph.D. supervisor, **Professor Nadimpalli Siva Kumar**. Without his enthusiasm, his inspiration, his guidance and persistent help, this dissertation would not have been possible. Throughout my work, he provided encouragement, sound advice, and lots of good ideas. I would have been lost without him. I thank him for trusting me and giving me freedom and responsibility in my work. Even in his busy schedules, he was always available for discussion throughout my research work.*

Special thanks are due to Prof. M. Ramanadham, Head, Department of Biochemistry, Prof. A.S. Raghavendra, Dean, School of Life Sciences and the former Heads and Dean Prof.T.Suryanarayana for making my research feasible with the excellent infrastructure and laboratory facilities.

I would like to express my sincere gratitude to my doctoral committee members Prof. K. V. A. Ramaiah and Dr. Bramanandam, for assessing my research work in between and for the useful discussions.

I wish to express my cordial appreciation to Dr. Mrinal K. Bhattacharya for his valuable suggestions and advice.

I am grateful to Prof. P. Appa Rao and his research group particularly Sashidhar B and Debashish for the fruitful discussions and for allowing me to use their lab facilities.

I also express my sincere thanks to the entire faculty who taught me science in my M.Sc., which ultimately made me to choose research as my career.

I thank Prof. Regina Pohlmann for providing us the mpr$^{(-/-)}$ MEF cells and cathepsin D antiserum, Prof. Stefan Hoening for the Lamp 1 monoclonal antibody and Prof. Gieselmann for the Arylsulfatase A antibody.

I am also indebted to Dr. K. Suresh, for the valuable instructions, suggestions and his warm hospitality during my stay in Boston.

I am also grateful to the Central Instrumental Laboratory, University of Hyderabad for providing excellent research facilities. I thank Mr. Murthy, Miss. Ramadevi for their help in confocal microscopy, Mr. Lallan and other non-teaching staff members for their help and assistance.

I cannot finish without thanking all my former and present workmates: Dr. Lavanya Latha, Dr. Nagender Rao, Mr. Sivaramakrishna, Mr. Kiran, Miss. Merino Visa, Mr. Venu and lab attendant Mr. Ramana for maintaining a cheerful atmosphere in the lab. I express my gratitude to all the research scholars of the university, where my stay was filled with wonderful experiences.

And I am deeply grateful to the Council for Scientific and Industrial research, India for the financial support.

Travel award worth $1000 received by the '2007 Awards Committee of the Society for Glycobiology' to attend Society meeting in Boston is highly acknowledged.

I cannot end without thanking my family, on whose constant encouragement and love I have relied throughout this endeavor.

Lastly, and most importantly, I wish to thank my wife, Bhavani who always supported and encouraged me to do my best in all matters of life.

Praveen Kumar

CONTENTS

ABBREVIATIONS

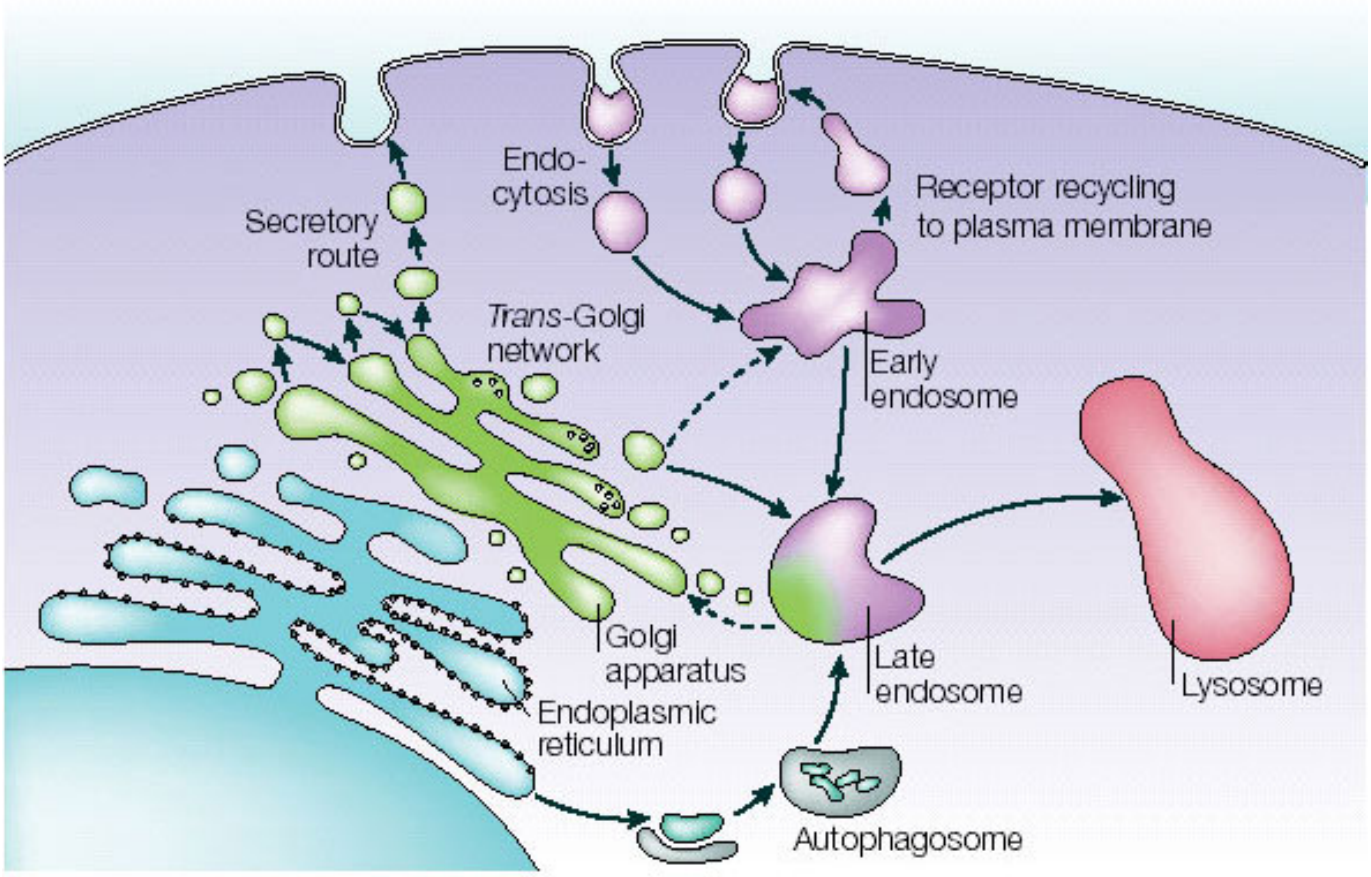
ALP	Alkaline phosphatase
AP1	Adaptor protein1
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bg	*Biomphalaria glabrata*
bp	base pair
BSA	Bovine serum albumin
CDMPR	Cation dependent mannose 6-phosphate receptor
cDNA	Complementary DNA
CEF	Chicken embryonic fibroblast
Ci	Curie
CIMPR	Cation independent mannose 6- phosphate receptor
cpm	Counts per minute
D-man	D-mannose
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DVS	Divinyl sulfone
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
ELISA	Ezyme-linked immunosorbent assay
EndoH	Endoglycosidase H
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FITC	Fluorescein-isothiocyanate
GGA	Golgi-localized, γ-ear-containing, ADP-

	ribosylation factor binding protein family
G6P	Glucose 6-phosphate
HEPES	(N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid))
IGF-II or IGF2	Insulin like growth factor-II
Im	Immuneserum
IP	Immunoprecipitation
kb	Kilo basepair
K_d	Dissociation constant
kDa	Kilo Dalton
Lamp-1	Lysosomal associated membrane protein1
LB	Luria Bertani
LSD	Lysosomal storage disorders
MEF	Mouse embryonic fibroblast
M6P	Mannose 6-phosphate
min	Minute
MPR	Mannose 6-phosphate receptor
NBT	Nitroblue tetrazolium
NC	Nitrocellulose
nm	Nanometer
nM	Nano molar
NP-40	Nonidet-P40
OD	Optical density
PACS	Phosphofurin acidic cluster-sorting protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/ Streptomycin
pH	-log (H^+) concentration
PI	Preimmuneserum
PM	Phosphomannan

pmol	Picomole
PMP	Pentamannosyl phosphate
PMSF	Phenyl methyl sulfonyl fluoride
PNGase F	Peptidyl N-glycosidase F
PPO	2,5-diphenyl oxazole
PVDF	Polyvinyl difluoride
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
SDS	Sodium dodecyl sulfate
Taq	*Thermophilus aquaticus*
TBS	Tris-buffered saline
TGN	*trans*-Golgi network
TIP47	Tail-interacting protein, 47 kDa
Tris	Tris-(Hydroxymethyl) aminoethane
µCi	micro Curie

CHAPTER 1

INTRODUCTION



Copyright © 2005 Nature Publishing Group
Nature Reviews | Neuroscience

1. INTRODUCTION

1.1. Lysosomes

Lysosomes present in all nucleated cells (discovered by Christian de Duve in 1959) are dense cytoplasmic vacuoles surrounded by a limiting membrane and are spherical, ovoid or occasionally tubular in shape. Their size ranges from 1 μm to several microns and are called the cells recycling plant, as they are involved in removing damaged and effete macromolecules from the cells environment and from within the cell itself, converting them into reusable products. This view of the lysosome has persisted until mid 1960's. The review of de Duve and Wattiaux (1966) was a significant milestone in setting lysosomes in a broader cell biology framework. The review also provided the first attempt at a classification of the various organelles comprising the vacuolar apparatus. The secondary lysosomes are formed by the fusion of primary lysosomes and the phagosome, which could be either an autophagosome or a heterophagosome. It was proposed that the autophagosomes were formed by the sequestration of cytoplasm by cellular membranes. In contrast the heterophagosome was derived from the plasma membrane and brought either by solutes (pinocytosis) or particles (phagocytosis) into the cell. Heterophagosomes that shuttled exogenous materials to lysosomes for digestion were later termed as endosomes. The endosome is now envisioned as highly dynamic structure that interchanges components with other endosomes, the Golgi complex and the plasma membrane through budding and fusion events (Beron et al., 1995). There is also a broad agreement that there are two subsets of endosomes; early (or light) endosomes and late (or dense) endosomes. The early endosome, containing ligands as well as solutes endocytosed in the bulk fluid phase, are transferred to the late endosome. The late endosome (or the prelysosome) is the organelle to which the lysosomal enzymes are delivered from their site of synthesis in the rough endoplasmic reticulum, following their exit from the Golgi apparatus and subsequently localized or found in Golgi-derived vesicles. In the endosomes, enzymes and substrates are together, both free from the receptors that deliver them. But because the particles internal pH may not be optimum for digestion and because the enzymes are not in their mature forms these structures are not

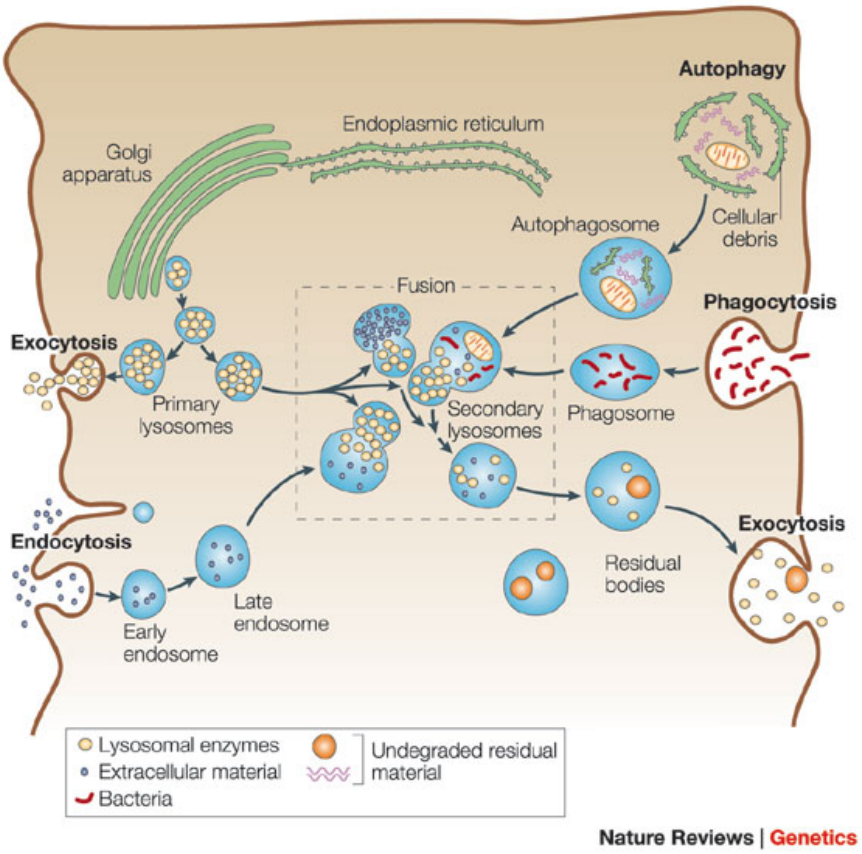


Figure 1.1. Different pathways leading to degradation in lysosomes

considered as lysosomes but are termed as prelysosomes. Some researchers maintain that late endosomes evolve into lysosomes. Beron et al. (1995) wrote that late endosomes progressively acquire lysosomal characteristics. There is, however, experimental evidence that the delivery of ligand from late edosome to lysosome involves a fusion event (Mullock et al., 1994). Lysosomes appear to be widely heterogeneous in nature with respect to internal osmolarity (Appelmans and de Duve, 1995). Lysosomes represent the major intracellular site for the degradation of a variety of macromolecules, including proteins, carbohydrates, nucleic acids and lipids which are internalized from the extracellular space by endocytosis, delivered by fusion with phagosomes or autophagosomes, or delivered from the biosynthetic pathway.

1.2. Lysosomal membrane

One crucial role of the membrane enclosing late endosomes and lysosomes is to separate the potent luminal acid hydrolases from other cellular constituents and so protect them from unwanted degradation. Cholesterol, dolichol derivatives, bis-

monoacylglycerol and phospholipids, including sphingomyelin are the major lipids of the 7-10 nm thick lipid-bilayer of the lysosomal membrane. Although the lysosomal membrane is usually impermeable to macromolecules and intermediate digestion products, it allows small molecules of up to 200 Da to diffuse freely. The lysosomal limiting membrane has multiple functions including acidification of the lysosomal matrix, sequestration of lysosomal enzymes, mediation of fusion events between lysosomes and other organelles, and transport of degradation products to the cytoplasm. About 50% of lysosomal membrane proteins comprise a class of acidic integral, highly glycosylated proteins decorating the luminal surface of lysosomal membranes. Lysosomal associated membrane protein 1 and 2 (LAMP-1, 107 kDa and LAMP-2, 110 kDa) which are type I membrane glycoproteins and lysosomal integral membrane protein 1 and 2 (LIMP-1, 35-55 kDa and LIMP-2, 60-85 kDa) which are type III membrane glycoproteins and are the most abundant components of this membrane.

1.3. Lysosomal storage disorders (LSDs)

According to GOLD (Global Organization for Lysosomal Diseases, established in Feb-2002), LSDs are a group of approximately 50 genetic (inherited) disorders, sharing common clinical and biochemical characteristics. Individually, each disease is rare, but by considering all LSDs as a common entity the prevalence is as 1:5000. Most of the LSDs are inherited autosomal recessive genetic defects (except Fabry and MPS II, which are X-linked recessive). Those who receive a mutated gene from only one parent are carriers, and generally have no or mild clinical manifestations. Symptoms of some lysosomal storage disorders were first identified as early as the 1880's and by the early 1900's, many LSDs have been described and classified-although the lysosome itself was not discovered until 1955. Many LSDs were identified before their biochemical and genetic basis was fully understood, so they received common names (for example, after the scientist discovering the disease). Later, an additional, more clinically descriptive name often came into use. By the 1960's the role of lysosomes in cellular digestion and substrate management was well understood, and Pompe's disease became the first disorder formally recognized as a lysosomal storage disease.

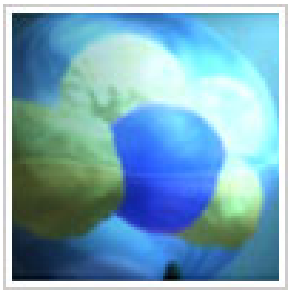


Figure 1.2. Healthy cell vs. LSD cell with accumulated substrate (source: http://www.lysosomallearning.com/healthcare/about/lsd_hc_abt_classification.asp#P60_3661)

By the 1970's, the scientific community had recognized many more LSDs as such and had begun identifying and classifying the specific enzymatic problems. To date about 50 different lysosomal disorders are now known in humans (LSDs). They result from the defective function of a specific protein, which leads ultimately to the progressive accumulation within the lysosome of either undegraded substrate(s) or catabolic products that are unable to escape from this organelle. Every LSD results from a problem with the lysosomal process by which enzymes digest the substrate. Lysosomes contain approximately 75 different lysosomal enzymes (including glucosidases, lipases, proteases and nucleases) and are involved in these degradative processes; since many of the substrates are complex lipids which are not water soluble, cofactors or activators may also be involved in enzyme-substrate interactions. Other proteins may be involved in protecting enzymes from being degraded themselves in this proteolytic environment. When a lysosomal enzyme (or another type of enzyme that directs it) is deficient or malfunctioning, the substrate it targets accumulates, interfering with normal cellular activity. The degradation of large macromolecules is achieved in a stepwise manner by removal of terminal residues by a series of lysosomal enzymes, usually exohydrolases, and the released monomeric units are either transported or diffuse out of the lysosome. A useful property of these lysosomal enzymes is the fact that although they are highly specific towards the structure and linkage of the terminal moiety on a complex macromolecule, the overall structure may not be important. It is therefore possible to measure lysosomal enzyme activities using a variety of relatively simple water-soluble substrates incorporating coloured or fluorescent groups. Such assays are commonly used for diagnostic studies. Defects in lysosomal enzymes,

cofactors or transport proteins may all give rise to specific lysosomal storage disorders and these can be classed conveniently according to the type(s) of storage material accumulating. LSDs can be classified further into sub-categories based on the type of enzymatic defect and/or stored substrate product. For example, the mucopolysaccharidoses (the “MPS” diseases) are grouped together because each results from an enzyme deficiency that causes accumulation of a particular glycosaminoglycan substrate. Following are the eight LSD sub-categories and the diseases that fall under them.

(Pictures taken from Semin Neonatol 2002; **7:** 75–83, ***Lysosomal disorders J. E. Wraith)***

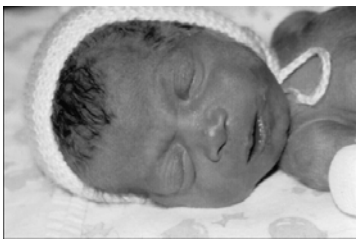


Fig 1.3. The facial dysmorphism associated with lysosomal storage disease. In this infant the underlying diagnosis is mucolipidosis II (I-cell disease), but this facial phenotype (Hurler-like) is also seen in GM1-gangliosidosis and infantile sialic acid storage disease

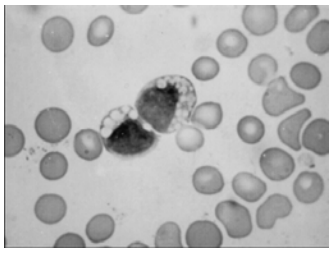


Fig 1.4. A peripheral blood film showing two prominent, vacuolated lymphocytes from an infant with α-mannosidosis.

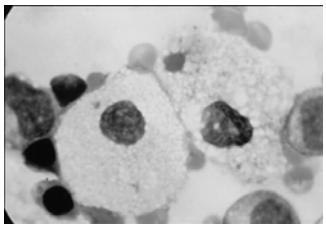


Fig 1.5. A bone marrow aspirate from an infant with Niemann–Pick A disease showing the characteristic foam cells.

Table 1.1. List of the stored product or enzymatic defect and resulting diseases.

Defective processes	Diseases
Metabolism of Glycosaminoglycans or mucopolysaccharidoses (MPS)	MPS I, II, III, IV, VI and VII
Degradation of glycan portion of glycoproteins	Aspartylglucosaminuria, fucosidosis type I and II, mannosidosis, sialidosis type I and II
Degradation of glycogen	Pompe disease
Degradation of sphingolipid components	Acid sphingomyelinase deficiency, Fabry disease, Farber disease, Gaucher disease type I, II and III, GM1 gangliosidosis type I, II and III, Tay-Sachs disease type I, II and III, Sandhoff disease, Krabbé disease, metachromatic leukodystrophy type I, II and III.
Degradation of polypeptides	Pycnodysostosis
Degradation or transport of cholesterol, cholesterol esters, or other complex lipids.	Neuronal ceroid lipofuscinosis type I, II, III and IV
Multiple deficiencies of lysosomal enzymes	Galactosialidosis, mucolipidosis II and III
Transport and trafficking defects	Cystinosis, mucolipidosis IV, infantile sialic acid storage disease (ISSD), Salla disease

1.3.1. Diagnosis and treatment

Diagnosis can be helped by appropriate radiological studies and, in some patients, evidence of the storage phenomena can be seen in peripheral blood smears or bone marrow aspirates. New developments in therapy including enzyme replacement therapy and substrate deprivation may improve prognosis in some disorders. Prenatal testing is often possible, most reliably through amniocentesis (sometimes also by chorionic villus sampling). Low levels of a particular enzyme confirm the LSD associated with that enzyme defect. Although, the samples are relatively easy to obtain from patients and analysis of the test samples takes only a few days, the results are usually complex to interpret and must be analyzed by a specialized laboratory. Enzyme assays are considered the most definitive test, but they are not available for every LSD. In some cases, other methods may be used, such as brain MRIs, electroretinogram, or biopsy of enlarged tissue. Mutation analysis can check for a gene mutation known to cause a particular disorder. It is not always conclusive, however, since there are usually

several mutations that may cause particular LSD, and a patient may have an unidentified mutation.

1.4. Mannose 6-phosphate targeting system

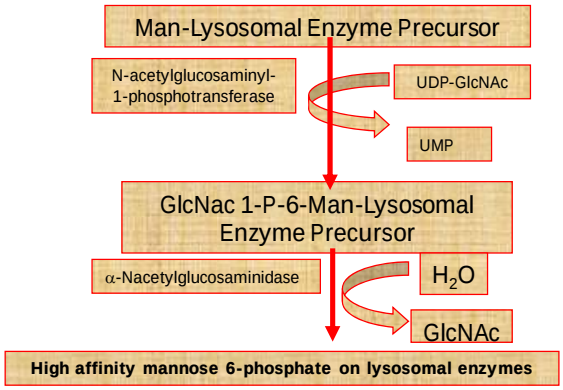
1.4.1. Identification of the targeting system

Leroy and DeMars in 1967, described a new disorder that clinically resembled Hurler syndrome (deficiency of α-L-Iduronidase leading to accumulation of deramatan and heparan sulphate), but lacked mucopolysaccharidiuria. Another distinguishing feature was the presence of large, phase-dense inclusions in the patient fibroblasts. Because these cells contained the inclusion bodies, the disorder came to be called as I-cell disease. This observation provided the initial basis towards identification of the lysosomal enzyme targeting system. By the early 1970's, other biochemical abnormalities were observed in I-cell disease. Cultured fibroblasts were found to be deficient for multiple acid hydrolases, many of which were present in abnormally high levels in culture medium (Lightbody et al., 1971; Tondeur et al., 1971). Similar inclusions were seen in pseudo-Hurler polydystrophy, which was clinically milder than I-cell disease and presented later (Kelly et al., 1975). In addition, the serum and body fluids of such patients showed elevated levels of these lysosomal enzymes. Hickman and Neufeld, (1972) made the key observations that I-cell fibroblasts were capable of endocytosis of acid hydrolases secreted by normal fibroblasts, but that the enzymes secreted in excess by I-cell fibroblasts were not subject to endocytosis by normal fibroblasts. Similar biochemical abnormalities were identified in MLIII (Glaser et al., 1974). These observations suggested that lysosomal enzymes contain a recognition marker for uptake and transport to lysosomes and that the enzymes secreted by I-cell fibroblasts lack this marker (Hickman et al., 1974). This hypothesis was subsequently confirmed, and the recognition marker was identified as mannose 6-phosphate in Prof. Sly's laboratory (Kaplan et al., 1977; Natowicz et al., 1979).

1.4.2. Biosynthesis of Mannose 6-phosphate (M6P) marker on lysosomal enzymes

The biosynthetic pathway by which the mannose 6-phosphate is added to newly synthesized acid hydrolases was elucidated in the laboratories of von Figura and

Hasilik, 1986 (Germany) and Kornfeld, 1987 (USA). The mechanism for processing and targeting acid hydrolases to lysosomes involves some of the same machinery used for synthesis of other N-linked glycoproteins. Lysosomal enzymes are synthesized on membrane-bound ribosomes and translocated to the lumen of the endoplasmic reticulum, where *N*-linked oligosaccharide chains are added (Kornfeld, 1987). After transfer to the Golgi apparatus, mannose 6-phosphate residues are added to acid hydrolases in a process that requires the sequential action of 2 enzymes. The first enzyme catalyzes the addition of an α-*N*-acetylglucosamine 1-phosphate residue to the 6 position of mannose on high-mannose oligosaccharide chains. The second enzyme removes *N*-acetylglucosamine to expose the mannose 6-phosphate. The "uncovering enzyme" was later characterized and shown to be a tetramer composed of 2 disulfide linked homodimers of 68 kDa subunits. To date, no disease has been attributed to a deficiency of this enzyme (Kornfeld et al., 1998). The following scheme shows the generation of mannose 6-phosphate residue on the lysosomal enzyme.



Cells from patients with I-cell disease and MLIII were shown to be deficient in the first enzyme in this pathway (UDP*N*-UDP-*N*- acetylglucosamine: lysosomal enzyme *N*-a cetylglucosamine 1-phosphotransferase).

These observations provided the basis for many subsequent studies that eventually led to the identification of the receptor proteins which recognize M6P marker. Delivery of ~50 different newly synthesized lysosomal enzymes to lysosomes is dependent upon their acquisition of mannose 6- phosphate (M6P) residues that act as recognition signals for high-affinity binding to the M6P receptors (MPRs), the ~46 kDa cation dependent MPR (CDMPR) and the ~300 kDa cation independent receptor/ MPR

300/insulin-like growth factor II receptor (CIMPR/MPR 300/IGF2R/) (Kornfeld, 1992; Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998). The two MPRs constitute the sole members of the P-type lectin family, a designation given based on the unique ability of these lectins to recognize phosphorylated mannose residues (Drickamer and Taylor, 1993).

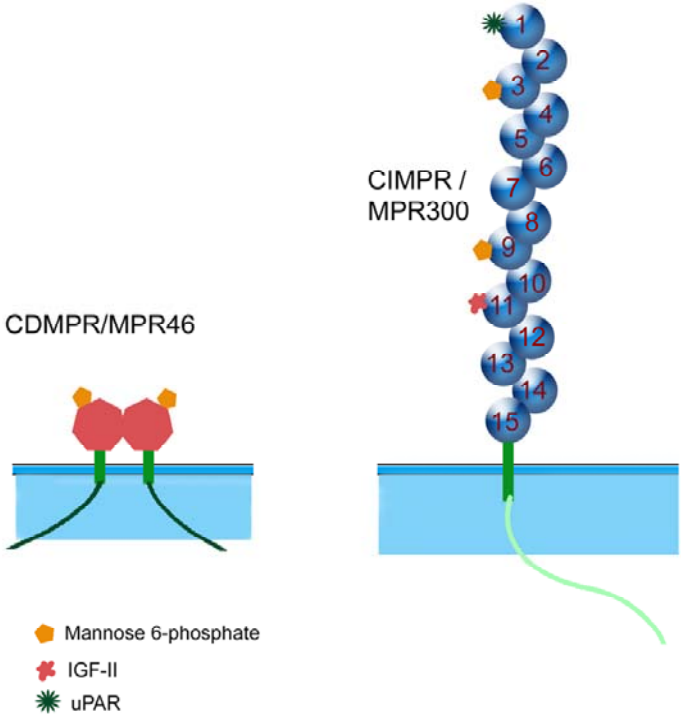


Figure 1.6. Arrangement of extracellular, transmembrane and cytosolic domains of MPR 46 and 300. The cation dependent mannose 6-phopshate receptor (CDMPR/MPR 46) exists predominantly as a stable homodimer in membranes and has a single mannose 6-phosphate (M6P) binding site per polypeptide. The cation independent mannose 6-phosphate receptor (CIMPR/MPR 300) seems to be a dimer in the membrane, although it acts as monomer in detergent solutions. MPR 300 has 15 repetitive domains in the extracytoplasmic region, domain 3 and 9 are involved in binding M6P, domain 5 also has low affinity towards M6P. Domain 11 binds to IGF-II (insulin like growth factor II protein), domain 1 is involved in binding to urokinase plasminogen activator receptor (uPAR).

1.4.3. Importance of MPRs

The importance of the two MPRs as major components of the M6P dependent transport system is further illustrated by the phenotype of primary embryonic fibroblasts devoid of both receptors (Ludwig et al., 1994). Such cells, generated by crossing CDMPR negative mice with ThP mice, secrete most of their newly synthesized lysosomal enzymes and as a consequence, accumulate undigested substrates in their late endocytic compartments. Thus, MPR negative fibroblasts have a phenotype very similar to that of fibroblasts from patients with mucolipidosis II unable to synthesize the M6P recognition marker on their lysosomal enzymes. Each MPR appears to carry out specific functions in this sorting process. This view is supported by the following

observations. First, massive over expression of the CDMPR in cells lacking the CIMPR receptor does not totally correct the hyper secretion of lysosomal enzymes (Ma et al., 1991; Johnson and Kornfeld, 1992). Second, although some hydrolases are secreted equally by both cell types, a few hydrolases are preferentially secreted by CDMPR negative fibroblasts while some others (including cathepsin D) are preferentially secreted by fibroblasts lacking the other MPR (Ludwig et al., 1994). Therefore, it would appear that the MPRs exhibit various affinities for different subgroups of lysosomal enzymes *in vivo.* Thus, the MPRs probably function in concert to sort the ~50 different hydrolases to lysosomes.

1.4.4. MPR 300/ IGF2R/CIMPR

1.4.4.1. Genomic structure and expression

Although the MPR 300/IGF2R is ubiquitously expressed in adult cells and tissues, a number of studies have demonstrated that the expression level of the IGF2R/MPR 300 is both tissue specific and developmentally regulated (Alexandrides et al., 1989; Wenk et al., 1991; Funk et al., 1992; Matzner et al., 1992; Nissley et al., 1993). The genomic structure of the MPR 300/IGF2R receptor has been analyzed for the mouse and the human. The MPR 300/IGF2R receptor gene in mouse is mapped to chromosome 17 (Laureys et al., 1988; Szebenyi and Rotwein, 1994), and in the human gene has been mapped to chromosome 6 (Laureys et al., 1988; Killian and Jirtle, 1999). The total size of the human MPR 300 gene is estimated to be 136 kb and comprises of 48 exons. Unlike other multidomain receptors, such as the human low-density lipoprotein receptor, the exon boundaries of the MPR 300 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 (Laureys et al., 1988; Killian and Jirtle, 1999). 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).

1.4.4.2. Primary structure of the MPR 300/IGF2R

Complete cDNA sequences of the MPR 300 have been obtained from a number of diverse sources as listed in Table 1.3. Partial cDNA sequences of the MPR 300 have also been obtained from sheep (NCBI accession no. AAG48349), kangaroo (Yandell et al., 1999), and the fish (Yerramalla et al., 2000). The MPR 300 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. 1.6) (Hille-Rehfeld, 1995; Korner et al., 1995; Braulke, 1999; Dahms and Hancock, 2002). The 13 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 (MacDonald and Czech, 1985; Kiess et al., 1991; Lobel et al., 1998; Braulke, 1999; Dahms and Hancock, 2002; Hassan, 2003). Cysteine residues located in the extracellular repeating segments of the receptor form intramolecular disulfide bonds required for proper receptor folding. Other posttranslational modifications, such as phosphorylation and palmitoylation have also been reported for the receptor (Westcott and Rome, 1988; Hille-Rehfeld, 1995; Dahms and Hancock, 2002). 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; Marron-Terada et al., 2000; Dahms and Hancock, 2002). The available data indicates that the receptor dimerization can occur both *in vitro* and *in vivo*. The extracellular domain can also bind other ligands such as IGF2 (discussed below).

1.4.4.3. Multi functional nature of MPR 300/IGF2R

Morgan et al. (1987) provided the first evidence, which unequivocally showed that the large mannose 6-phosphate receptor (275 to 300 kDa) was also a receptor for the insulin-like growth factor II (IGF2). 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 simultaneous binding of more than one ligand, alters the kinetics of MPR 300 receptor internalization at the cell surface (York et al., 1999; Byrd et al., 2000; Dahms and Hancock, 2002; Hassan, 2003). Since then, investigation of this multifunctional molecule has continued to reveal interesting information, which extends its relevance across a wide range of biological and pathological processes, from genomic imprinting, human intelligence, to tumor suppression.

Cell surface CIMPR receptors, but not CDMPR receptors, mediate endocytosis of a variety of M6P-containing ligands for their subsequent clearance or activation. The CIMPR 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 CDMPR receptor (Koster et al., 1994; Hille-Rehfeld, 1995). Endocytosis of lysosomal enzymes by the CIMPR receptor serves as a mechanism to facilitate degradation of extracellular matrix proteoglycans or to transfer enzymes from one cell type to another (Brauker et al., 1983; Roff et al., 1983). The MPR 300/IGF2R receptor binds M6P-containing ligands and IGF-II at two distinct sites (Nissley and Kiess, 1991; Kornfeld, 1992; Hille-Rehfeld, 1995; Braulke, 1999; Dahms and Hancock, 2002). The M6P binding sites localize to repeats 1–3 and 7–11 of the extracytoplasmic receptor region. Equilibrium dialysis experiments have demonstrated that the receptor binds 2 moles of M6P or 1 mole of β-galactosidase or equivalent lysosomal enzymes via their M6P-residues (Tong et al., 1989; Distler et al., 1991; Westlund et al., 1991; Hille-Rehfeld, 1995; Dahms and Hancock, 2002). In addition to lysosomal enzymes, this receptor also binds a diverse spectrum of other M6Pcontaining proteins, as well as non-M6P-containing ligands as listed in Table 1.2. Site directed mutagenesis studies combined with pentamannosyl phosphate agarose chromatography and binding affinity analyses, have identified five amino acid residues in both domain 3 (Q392, S431, R435, E460 and Y465) and domain 9 (Q1292, H1329, R1334, E1354 and Y1360), which are essential for carbohydrate recognition by the bovine MPR 300 receptor (Dahms et al., 1993; Dahms and Hancock, 2002; Hancock et al., 2002). The carboxy-terminal M6P binding site located on domain 9 of the MPR 300 receptor exhibits optimal binding at pH

6.4–6.5, whereas the amino-terminal M6P binding site of domain 3 demonstrates a higher optimal binding pH of 6.9–7.0. Additionally, binding inhibition experiments have also demonstrated that the carboxy-terminal M6P binding site of the receptor is highly specific for the phosphomonoester and M6P, whereas the amino-terminal carbohydrate recognition site can efficiently bind M6P-OCH3 phosphodiester and mannose-6-sulfate with a slightly lower affinity than M6P (Marron-Terada et al., 2000; Dahms and Hancock, 2002). These results suggest that the M6P sites of the MPR 300 receptor can be bound not only by ligands with a relatively broad pH range, but also can recognize a great diversity of ligands. The ability of the CIMPR 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.

Table 1.2. List of M6P containing and non-M6P containing ligands that bind to MPR 300/IGF2R

Reported ligands for the MPR 300/IGF2R

M6P-containing ligands	Consequence of binding to the IGF-II/MPR	References
Lysosomal enzymes	Endocytosis and/or targeting to lysosomes	(Kornfeld and Mellman, 1989; Kornfeld, 1992; Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001)
Transforming growth factor-β precursor (TGF- β)	Proteolytic activation at the cell surface	(Dennis and Rifkin,1991; Jirtle et al., 1991; Munger et al., 1997; Purchio et al.,1988; Godar et al., 1999; Ghahary et al., 1999, 2000)
Leukemia inhibitory factor	Endocytosis and degradation in lysosomes	(Blanchard et al., 1998, 1999)
Proliferin	Induction of endothelial cell migration and angiogenesis	(Volpert et al., 1996; Groskopf et al., 1997; Jackson and Linzer, 1997; Lee and Nathans, 1988)
Thyroglobulin	Endocytosis and activation and/or degradation in lysosomes?	(Herzog et al., 1987; Scheel and Herzog, 1989; Kostrouch et al., 1991; Lemansky and Herzog, 1992)
Renin precursor	Internalization and proteolytic activation and/or degradation in lysosomes	(Faust et al., 1987; Aeed et al., 1992; van Kesteren et

		al., 1997; Admiraal et al., 1999; Danser et al 1999; Saris et al., 2001a, 2001b; van den Eijnden et al., 2001)
Granzyme A	Targeting to lytic granules and role in apoptosis?	(Burkhardt et al., 1989; Griffiths and Isaaz, 1993)
Granzyme B	Internalization and rapid induction of apoptosis	(Griffiths and Isaaz, 1993. Motyka et al., 2000)
DNase I	Targeting to lysosomes?	(Frenz et al.,1994; Nishikawa et al., 1997; Cacia et al., 1998; Nishikawa et al., 1999)
CD26	Internalization and role in T cell activation	(Ikushima et al., 2000; Ohnuma et al., 2001)
Epidermal growth factor receptor	Endocytosis and degradation in lysosomes?	(Todderud and Carpenter, 1988; Babst et al., 2000)
Herpes simplex viral glycoprotein D	Facilitation of viral entry into cells and transmission between cells	(Brunetti et al., 1994, 1995; Topp et al., 1997; Brunetti et al., 1998)
Varicella-zoster viral glycoprotein I	Facilitation of viral entry into cells	(Gabel et al., 1989; Zhu et al., 1995; Alconada et al., 1996; Wang et al., 2000)
Non M6P-containing ligands	Consequence of binding to the IGF-II/MPR	References
Insulin-like growth factor II (IGF-II)	Endocytosis and degradation in lysosomes	(Oka et al., 1985; Morgan et al., 1987; MacDonald et al., 1988; Tong et al., 1988; O'Dell and Day, 1998)
Retinoic acid	Growth inhibition and/or induction of apoptosis	(Kang et al., 1997, 1998, 1999)
Urokinase-type plasminogen activator receptor (uPAR)	Role in activation of TGF-h precursor at the cell surface; endocytosis and degradation in lysosomes	(Nunes et al., 1995; Nykjaer et al., 1998; Godar et al., 1999)
Plasminogen	Conversion to plasmin, which can activate TGF-h precursor	(Godar et al., 1999)
Heparinase	Extracellular matrix degradation	(Robert and Mark, 2008)

1.4.4.4. IGF-II recognition by the receptor

IGF-II is the best characterized non M6P-bearing ligand of CIMPR. This hormone plays key roles in metabolic regulation and fetal growth (Han et al., 1987) through three

types of receptors, two tyrosine kinase receptors (IGF-I receptor and insulin receptor isoform A) and CIMPR. The stimulation of the type 1 receptor tyrosine kinase induces a protein phophorylation cascade leading to biological effects, particularly the insulin-mediated growth and increase in CIMPR expression (Kandror and Pilch, 1988), which mediates endocytosis and clearance of IGF-II (Oka et al., 1985). The lysosomal degradation of IGF-II is critical because elevated levels of IGF-II induce overgrowth. Indeed, mice without CIMPR accumulate high amounts of IGF-II and die perinatally (Ludwig et al., 1993; Lau et al., 1994; Wang et al., 1994). As this phenotype can be reversed by a deficiency in IGF-II, it is directly caused by the over-expression of IGF-II. In humans, the soluble levels of CIMPR in serum are higher in infants and fall by 40% in adult life to reach a circulating level of about 700 µg/l (Bobek et al., 1992; Costello et al., 1999). The receptor's circulating level increases during pregnancy, diabetes, and appears correlated with the elevation of circulating lysosomal enzymes in both cases (Bobek et al., 1992). Though CIMPR circulates at a concentration lower than that of IGF-II, it seems to play a role in IGF-II clearance. It has been demonstrated that this soluble receptor exerts biological effect, notably by binding IGF-II and blocking IGF-II stimulated DNA synthesis in hepatocytes and fibroblasts, and at physiological concentrations this receptor can block tumour growth mediated by IGF-II (Scott and Weiss, 2000).

The IGF2R receptor from viviparous mammals binds IGF-II at a site localized to the amino-terminal portion of extracytoplasmic domain 11 (Dahms et al., 1994; Garmroudi and MacDonald, 1994; Schmidt et al., 1995). To date, mutagenesis studies have implicated only a single residue at 1572 in domain 11 as critically important for IGF-II binding - substitution of isoleucine with threonine at position 1572 eliminates IGF-II binding (Garmroudi et al., 1996). Although the primary determinants of binding reside in domain 11, the sequence elements within domain 13 have been suggested to contribute a ~5–10 fold enhancement to the binding affinity of the receptor for IGF-II (Devi et al., 1998; Linnell et al., 2001; Brown et al., 2002). Interestingly, studies of IGF2R receptor purified from opossum (Dahms et al., 1993) and kangaroo (Yandell et al., 1999) have indicated that marsupials, unlike opossum, exhibit lower binding affinities for IGF-II, whereas no significant IGF-II binding was observed for the IGF2R

receptor from platypus (Killian et al., 2000), chicken (Clairmont and Czech, 1989; Yang et al., 1991) or frog (Clairmont and Czech, 1989). This has been attributed to significant alterations in the amino acid sequence in the amino-terminal portion of domain 11 as compared to viviparous mammals (Dahms and Hancock, 2002). Although these findings suggest that IGF-II binding by the IGF2R receptor is confined to viviparous mammals, while the carbohydrate recognition function of the receptor is widely utilized by mammalian as well as non-mammalian species, a recent study on fish and chicken has provided the first evidence of IGF-II binding to the IGF2R receptor from these non-mammalian vertebrate species (Mendez et al., 2001; Suresh et al., 2006). Thus, the extent with which a functional IGF-II binding site is expressed in the IGF2R receptor among various species that are known to constitutively express the receptor remains to be fully defined. Additionally, a recent study in our laboratory also suggested that the reptilian receptor also binds IGF-II (Sivaramakrishna et al., 2008, under revision)

1.4.5. MPR 46/CDMPR

The function of the CDMPR in lysosomal enzyme transport was unclear until cells lacking this receptor were generated by gene disruption experiments in mice (Köster et al., 1993; Ludwig et al., 1993). This approach demonstrated that the CDMPR also functions in transport of hydrolases to lysosomes. Like cells lacking the MPR 300/IGF2R/CIMPR receptor, thymocytes or skin fibroblasts isolated from animals homozygous for a disrupted, non-functional CDMPR gene missort a proportion of their newly synthesized lysosomal enzymes. In CDMPR negative animals, these secreted phosphorylated ligands, some of which are enriched in extracellular fluids, are most likely endocytosed by the existing MPR 300 receptor, thereby explaining why these animals have a normal content of functional hydrolases in their tissues and are apparently normal and fertile. It has been reported for MPR 300/IGF2R-deficient rat Morris 7777 hepatoma cells, where treatment with blocking antibodies against MPR 46 induced the secretion of the majority of the residual intracellular acid hydrolases (Stein et al., 1987b). Similar results were obtained for the intracellular targeting of cathepsin B and other lysosomal proteinases in MPR 300/IGF2R negative murine fibroblasts and SCC-VII cells which relies on the presence of MPR 46 (Olivia et al., 2006). This

supports the concept that at least in the cell types investigated, biosynthetic transport of acid hydrolases to lysosomes strictly requires the presence of the M6P recognition marker and the expression of at least one of the two MPR receptors.

1.4.5.1. Genomic structure and expression of the CDMPR

The human CDMPR gene has been mapped to chromosome 12 (Pohlmann et al., 1987) and is comprised of seven exons that span a 12 kb region. The genomic structure of the CDMPR has been analyzed for the human (Klier et al., 1991) and the mouse (Ludwig et al., 1992). The mouse CDMPR gene is located on chromosome 6 (Ludwig et al., 1992). Unlike other multidomain receptors such as the human low-density lipoprotein receptor (Sudhof et al., 1985), the exon boundaries of the CDMPR do not correspond to its functional or structural domains: exon 1 encodes the 5' untranslated sequence; the signal sequence is encoded by a portion of exon 2; the extracytoplasmic region of the CDMPR is encoded by exons 2–5; portions of exons 5 and 6 encode the transmembrane domain; the cytoplasmic domain is encoded by segments of exons 6 and 7; and exon 7 also encodes the 3' untranslated sequence. With the exception of cells from MPR-deficient mouse mutants and certain tumor-derived cell lines (Gabel et al., 1983; Barlow et al., 1991; Ludwig et al., 1993, 1994, 1996; Wang et al., 1994; Pohlmann et al., 1995; Kasper et al., 1996; Dittmer et al., 1998), the MPRs are ubiquitously expressed in mammalian cells and tissues. Analyses of the promoter region of the human CDMPR gene identified features characteristic of promoters for housekeeping genes, suggesting that the CDMPR gene is constitutively expressed in human tissues (Klier et al., 1991). However, quantitative analyses of various human tissues and cell lines using a highly specific enzyme-linked immunosorbent assay revealed cell-type and tissue-specific differences in the steady-state concentrations of the receptor (Wenk et al., 1991). Furthermore the mRNA levels, during development and in adult tissues of mice and chicken also vary (Ludwig et al., 1992; Matzner et al., 1992, 1996). Thus, several lines of evidence indicate that CDMPR expression levels are not only developmentally regulated but also are tissue specific.

1.4.5.2. Primary structure of the CDMPR

To date, amino acid sequence information for the CDMPR has been obtained from cDNAs cloned from several species as listed in Table 1.3. The ~46 kDa CDMPR is a type I transmembrane glycoprotein composed of four functional/structural domains. The amino-terminal signal sequence, comprised of 28 amino acids in the bovine CDMPR, directs translocation of nascent CDMPR polypeptides across the ER membrane and is subsequently cleaved. The 159-residue extracytoplasmic region harbors the M6P binding site. Anchoring the receptor to the membrane is a 25 residue single-pass transmembrane domain. Finally, the 67 residue carboxyl-terminal sequence extends into the cytosol where it functions in receptor trafficking.

1.4.5.3. Biosynthesis, co- and post-translational modifications of the CDMPR

Newly synthesized CDMPR polypeptides undergo cotranslational ER membrane insertion and core glycosylation at four out of the five potential N-linked glycosylation sites located in its extracytoplasmic region. Several *in vitro* (Hoflack and Kornfeld, 1985; Hille et al., 1989) and *in vivo* (Wendland et al., 1991; Zhang and Dahms, 1993; Hille et al., 1990) studies have been performed to ascertain the functional significance of the N-linked oligosaccharides, which together account for nearly 20% of the total mass of the CDMPR. The consensus from these studies is that the N-linked oligosaccharides do not play a direct role in ligand binding, an observation that has been confirmed by recent crystallography studies (Roberts et al., 1998; Olson et al., 1999). Furthermore, they do not appear to be essential for the intracellular stability and subcellular distribution of the CDMPR. Instead, the presence of N-linked oligosaccharides appears to promote the proper folding of the receptor into its native conformation, which is a prerequisite for high-affinity ligand binding and intracellular transport. Significantly, a truncated, glycosylation-deficient CDMPR mutant lacking three of the four N-linked oligosaccharides retains binding affinity nearly identical to the native CDMPR (Marron-Terada et al., 1998), indicating that a single N-linked oligosaccharide is sufficient to facilitate proper folding. In addition to N-linked glycosylation sites, the CDMPR contains six cysteine residues in its extracytoplasmic region that are used to generate three intramolecular disulfide bonds (Roberts et al., 1998), constituting a critical early step in

the formation of properly folded receptors (Hille et al., 1989; 1990; Li et al., 1990; Wendland et al., 1991). Replacement of any of the six cysteine residues with glycine disrupts the proper folding of the CDMPR and results in a complete loss of M6P binding ability (Wendland et al., 1991). Following initial disulfide bond formation, a conformational change occurs in the CDMPR such that increased trypsin resistance and immunogenicity as well as acquisition of ligand binding ability is observed (Hille et al., 1989, 1990). The precise molecular mechanism responsible for this conformational change has not been determined, although rearrangement of the disulfide bonds has been proposed to be a key factor (Hille et al., 1989; Olson and Lane, 1989). Assembly of the CDMPR into noncovalent oligomeric forms accompanies the generation of its ligand binding conformation (Hille et al., 1989, 1990) and is influenced *in vitro* by factors such as receptor concentration, pH, temperature, and the presence of divalent cations and ligands (Li et al., 1990; Waheed et al., 1990; Waheed and von Figura, 1990). Monomeric, trimeric and tetrameric forms of the CDMPR have also been observed, the CDMPR exists primarily as a homodimer in membranes (Hoflack and Kornfeld, 1985; Stein et al., 1987; Dahms and Kornfeld, 1989; Wendland et al., 1989; Li et al., 1990; Waheed et al., 1990; Waheed and von Figura, 1990; Ma et al., 1992; Punnonen et al., 1996). Biochemical studies of truncated forms of the CDMPR lacking the transmembrane and cytoplasmic domains demonstrated that the structural and functional information necessary for dimerization and high-affinity ligand binding is contained entirely within the extracytoplasmic region of the receptor (Wendland et al., 1989; Marron-Terada et al., 1998). Many investigators by *in vivo* studies provided several lines of evidence using human and mouse fibroblasts to show that neither receptor trafficking nor ligand binding and dissociation alter the oligomeric state of the membrane bound receptor (Punnonen et al., 1996). Instead, it was concluded that binding and dissociation of ligands is probably regulated by conformational changes in the receptor rather than by alterations in receptor oligomerization. This hypothesis is supported by the findings of several significant conformational differences between the ligand-bound and ligand-free dimeric structures of the CDMPR (Roberts et al., 1998; Olson et al., 1999; 2002). Together, the available biochemical and structural data indicate that the CDMPR exists and functions predominantly as a dimer within the cell.

The carboxyl-terminal cytoplasmic tail of the CDMPR is also subject to post-translational modifications, including phosphorylation and acylation, which are predicted to have a role in modulating the trafficking of the receptor. Both *in vivo* (Hemer et al., 1993) and *in vitro* (Korner et al., 1994) studies have demonstrated that phosphorylation occurs at a single serine residue, corresponding to position 57 in the carboxyl-terminal domain of the bovine CDMPR. Acylation of the CDMPR involves the reversible palmitoylation of the two cysteine residues, corresponding to positions 30 and 34 of the bovine receptor, in its cytoplasmic domain (Schweizer et al., 1996). It was predicted that palmitoylation might anchor the cytosolic tail to the membrane and thereby form a signaling loop necessary for normal lysosomal enzyme sorting and receptor trafficking (Schweizer et al., 1996). Consistent with this hypothesis, two aromatic residues (phenylalanine 18 and tryptophan 19) were identified as the key components of the putative endosomal signaling loop (Schweizer et al., 1997). Furthermore, insertions or deletions of multiple amino acids flanking the diaromatic residue motif resulted in the rapid degradation of the CDMPR, suggesting that the correct length and/or conformation of the putative signaling loop is an important factor, in addition to palmitoylation, to prevent targeting of the CDMPR for degradation (Schweizer et al., 1997; Breuer and Braulke, 1998).

1.4.6. Subcellular localization and trafficking of the MPRs

Several agents including growth factors, enzymes and chemical compounds have been shown to modulate cellular recycling and routing of the IGF2R/MPR 300 receptor. In human fibroblasts, a rapid and transient redistribution of MPR 300 receptors from internal pools to the cell surface is induced by IGF-I, IGF-II and epidermal growth factor. This redistribution is associated with a 2–3 fold increase in the binding and uptake of exogenous lysosomal enzymes (Braulke et al., 1989, 1990; Damke et al., 1992). Several kinases and phosphatases have been proposed to participate in the translocation and redistribution of cellular MPR 300 receptors (Kiess et al., 1994).

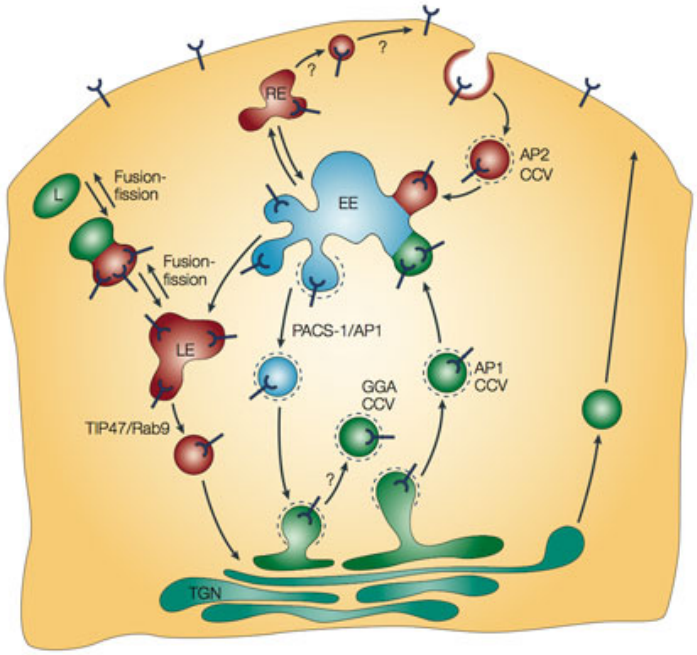


Figure reproduced from Nat. Rev. Mol. Cell Biol. (2003) 4; 202-212. Ghosh et al.

Figure 1.7. A schematic representation of the subcellular localization and trafficking itinerary of the MPRs. Mannsoe 6-phosphate receptors (MPRs) bind to their cargo (acid hydrolases) in the trans-Golgi network (TGN), and are pacakged into transport carriers athat deliver the receptor with its bound ligand to early endosomes (EE). The low pH within the endosomes facilitates the dissociation of the acid hydrolases from the MPRs. Dynamic fusion/fision between the late endosomal and lysosomal compartments results in selective delivery of the hydrolases to the lysosomes (L) (the 'Kiss-and-run' theory). TIP47/Rab9 prevent the MPRs from reaching the lysosomes, in which they would otherwise be degraded. The return pathway from the early endosomal compartment to the Golgi is probably mediated by PACS-1 assisted packaging into AP1-containing clathrin-coated vescicles (CCVs), whereas that from the late endosomal (LE) compartments is mediated by TIP47 and Rab9. Some of the MPRs go to the cell surface either from early or late endosomes through the recycling endosomes (RE), or from proximal TGN cisternae as a consequence of mis-sorting. The cell-surface receptors are internalized in AP2 CCVs and delivered back to the endosomes.

Recognition of the M6P signal found on newly synthesized acid hydrolases by MPRs occurs in the *trans*-Golgi network (TGN) (Le Borgne and Hoflack, 1998; Rohrer and Kornfeld, 2001), thereby selectively sorting the lysosomal enzymes from other proteins in the secretory pathway (Fig. 1.7). The enzyme-bound receptor complexes are subsequently transported from the TGN to endosomal compartments via clathrin-coated vesicles. Previously, interactions between the cytosolic tail of the MPRs and adaptor protein 1 (AP-1) complexes in conjunction with ADP-ribosylation factor were thought to mediate clathrin-coat assembly on vesicles budding from the TGN (Le Borgne and Hoflack, 1998; Dell'Angelica and Payne, 2001; Mullins and Bonifacino, 2001). Although a role for AP-1 in TGN-to-endosome trafficking of the MPRs has not been ruled out, several studies have provided strong evidence that members of the clathrin-associated Golgi-localized, γ-ear-containing, ADP-ribosylation factor binding (GGA) protein family, rather than AP-1, bind to the TGN sorting signal present in the cytosolic tails of the receptors (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001). In both MPRs, the TGN sorting signal is comprised of a cluster of predominantly acidic residues followed by a dileucine motif found near the carboxyl-terminus of their cytoplasmic

domain (Fig. 1.8) (Johnson and Kornfeld, 1992a, 1992b; Chen et al., 1997) that is recognized by the VHS (VPS27/Hrs/STAM) domain of the GGA family members (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001). Within the low-pH (< 6) environment of late endosomal/ prelysosomal compartments, the lysosomal enzymes are released from the MPRs (Fig. 1.7) and are further packaged into lysosomes by an unknown mechanism (Le Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001). The MPRs do not travel to lysosomes, but rather recycle from endosomal compartments back to the TGN to recruit additional M6P-containing acid hydrolases. Significantly, it has been shown for the CDMPR that a diaromatic endosomal sorting signal, comprised of phenylalanine 18 and tryptophan 19 in the cytosolic tail (Fig. 1.8), prevents its mislocalization to lysosomes (Schweizer et al., 1997). In previous studies by Pfeffer and

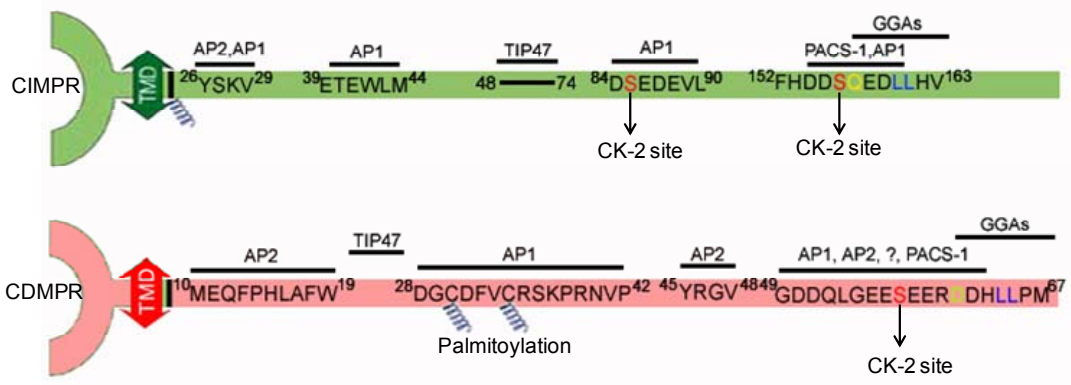


Figure reproduced from Nat. Rev. Mol. Cell Biol. (2003) 4; 202-212. Ghosh et al.

Figure 1.8. Sorting signals on MPR tails. A schematic representation of the cytosolic tails of the mannose 6-phosphate receptors (MPRs), which shows the identified amino-acid sorting signals and their associated transport proteins. The bars indicate the residues that are important for the specific binding partner. The residues are numbered from 1 to 163 in the cation-independent MPR (CIMPR), and from 1 to 67 in the cation-dependent MPR (CDMPR), starting from the transmembrane domain (TMD). The casein kinase 2 (CK-2) sites and palmitoylation sites are marked at the specific residues. GGA, Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding protein; PACS-1, phosphofurin acidic cluster-sorting protein; TIP47, tail-interacting protein, 47 kDa.

colleagues, using an *in vitro* transport assay, a late endosome-to-TGN transport of CDMPR mediated by TIP47 (tail interacting protein) and the GTPase Rab9 was reported in CHO cells (Itin et al., 1997; Diaz and Pfeffer, 1998). Later on by similar *in vitro* transport assay with membrane preparations from mouse embryonic fibroblasts (MEFs) by Guruprasad and Peter Schu, (2003) it was shown that in mouse fibroblasts, the retrograde transport of MPR 46 is brefeldin A sensitive and does not require TIP47. An explanation is that CHO cells transport the majority of CDMPR along a late endosome-to-TGN pathway, which is TIP47-dependent, whereas in mouse fibroblasts the majority of MPR 46 is transported along a direct early endosome-to-TGN pathway. The region of the MPR 300 that is recognized by TIP47 is highly conformational dependent and localized to residues 48–75 of the cytosolic tail of the human MPR 300 (Fig. 1.8) (Orsel et al., 2000). Recent work by Tikkanen et al. (2000) indicated that the carboxyl-terminal dileucine motif in the cytosolic tail of the CDMPR, which is involved in TGN-to-endosome trafficking, also functions to direct recycling of the receptor back to the TGN. The binding factors that recognize the dileucine motif for endosome-to-TGN trafficking have not been determined, although *in vitro* analyses appeared to rule out recognition of the dileucine motif by either the AP-1 or AP-2 complexes (Honing et al., 1997; Storch and Braulke, 2001). Nevertheless, the inability of AP-1 to bind the TGN sorting signal (Hogg et al., 1994; Zhu et al., 2001) coupled with recent data by Meyer et al. (2000, 2001), which demonstrated a significant disruption in the ability of the receptors to recycle back to the TGN in AP-1 deficient cells, suggest that AP-1 may play an important role in endosome-to-TGN receptor trafficking rather than sorting at the TGN. Interestingly, recycling of the MPR 300 back to the TGN was similarly impaired in experiments that disrupted the availability of functional phosphofurin acidic cluster sorting protein 1 (PACS1), a protein that binds *in vitro* to the carboxyl-terminal cluster of acidic residues in the cytosolic tail of the MPR 300 in addition to interacting with AP-1 (Wan et al., 1998; Crump et al., 2001). Together these findings led to the suggestion that PACS1 may act as a connector between the MPRs and AP-1 to facilitate recycling of the receptors to the TGN (Crump et al., 2001). Additional *in vitro* experiments have shown that the initial rate of endosome-to-TGN trafficking of the MPRs is enhanced by the presence of cytoplasmic dynein, mapmodulin, and polymerized microtubules,

supporting a role for the microtubule-based cytoskeleton in enhancing vesicular transport (Itin et al., 1999). The role of the CDMPR at the surface remains unclear, although results from studies that over expressed the CDMPR in hamster kidney epithelial cells and mouse L cells suggest that its trafficking to the cell surface may be a mechanism for regulated secretion of M6P-containing ligands into the extracellular milieu (Chao et al., 1990). The identity of the signal(s) in the cytosolic domains of the MPRs involved in directing them to the plasma membrane remains obscure, although a correlation between the phosphorylation status of specific serine residues and cell surface expression of the receptors has been observed (Hu et al., 1990; Braulke and Mieskes, 1992; Breuer et al., 1997; Zhang et al., 1997). However, in recent experiments, Nakagawa et al. (2000) demonstrated that a novel microtubule-dependent motor from the kinesin superfamily of proteins, KIF13A, transports MPR-containing vesicles to the plasma membrane via interactions of KIF13A with the AP-1 complex. Endocytosis of the MPRs, on the other hand, appears to primarily involve formation of clathrin-coated vesicles mediated by the AP-2 complex (Pearse, 1988; Glickman et al., 1989; Le Borgne and Hoflack, 1998). A single tyrosine-based internalization sequence, YKYSKV (residues 24–29), has been identified in the cytoplasmic domain of the bovine MPR 300 (Fig. 1.8) (Lobel et al., 1989; Canfield et al., 1991; Jadot et al., 1992) and evidence for AP-2 binding at this sequence has recently been presented (Orsel et al., 2000). In contrast, the cytosolic tail of the human CDMPR contains three separate internalization sequences: a phenylalanine-containing sequence, AKGMEQF (residues 7–13); a tyrosine-based motif, YRGV (residues 45–48); and the carboxyl-terminal dileucine (residues 64 and 65) motif that also influences sorting at the TGN and endosomes (Denzer et al., 1997). Consistent with the findings of multiple trafficking signals present in the CDMPR, *in vitro* biosensor studies and yeast two-hybrid experiments indicate that recognition of the cytosolic tail of the CDMPR by the heterotetramer AP-1 and AP-2 complexes involves multiple sequence elements (Honing et al., 1997; Storch and Braulke, 2001). Two of the CDMPR sequence elements bound by AP-2 include the AKGMEQF and the YRGV internalization signals, whereas the other major AP-2 binding region corresponds to the carboxyl-terminal cluster of acidic residues involved in TGN-to-endosome trafficking (Honing et al., 1997; Storch and

Braulke, 2001). Like AP-2, AP-1 binds to this cluster of acidic residues in addition to binding to a proximal region of the CDMPR tail encompassing residues 27–43 (Fig. 1.8) (Honing et al., 1997). Nevertheless, it is becoming increasingly apparent that biophysical techniques such as NMR or protein crystallography will be needed to more precisely define the multiple endocytosis and sorting signals of the MPRs and their conformational presentations to the various transport factors.

Table 1.3. M6P/IGF-II receptor vs. Cation-Dependent M6P receptor

Character	**MPR 46**	**MPR 300**
Chromosome location	12 (human), 6 (mouse)	6 (Human), 17 (mouse)
cDNA Cloning	12 kb, 7 exons in total	130 kb, 48 exons in total
	1. Human (Pohlmann et al., 1987) 2. Bovine (Dahms et al., 1987) 3. Murine (Köster et al., 1991., Ludwig *et al*., 1992; Ma et al., 1992) 4. Goat (Suresh et.al., 2004) 5. Chicken (praveen et al., 2008) 6. Fugu and *Xiphophorus* (*foot note) 7. Zebra fish (Suresh et al., 2005) 8. Starfish (Siva Ramakrishna and Siva kumar, 2008) 9. Xenopus tropicalis (BC081319XX) 10. killifish (Fundulus heteroclitus) the partial sequence DN951807	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 (*foot note) 8. Zebra fish (Catherine et al., 2006) 9. Pig, rabbit, bat, colugo, hedgehog, ring-tailed lemur, tree shrew, opossum, red-necked wallaby, echidna, and duckbill (Killian et al., 2001)
Deduced amino acid sequence(Human MPR 46)	277 amino acids in total	2491 amino acids in total
Signal sequence		
Extracytoplasmic domain	20-26	40
Transmembrane domain	164-170	2264
Cytoplasmic domain	20	23
Internal repeats within extracytoplasmic domain	67 1	164 15 (~ 147 amino acids each)
M_r of polypeptide	30 kDa	270 kDa
Apparent M_r	43-46 kDa	275-300 kDa
Post-translational Modifications		
N-glycosylation sites	5	19
Potential sites	2 high mannose	
glycosylated sites	2 complex	

Disulfide bonds	3 pairs	3-4 pairs per repeat
Oligomerization	dimers, tetramers	monomer, oligomer ?
Phosphorylation	ser 56 (CK II)	ser 82 (CK II), ser 157 (CK II)
Palmitoylation	Yes	Yes
Binding characteristics		
Requirement for divalent cations for optimal binding	Required	Not required
Binding other non-glycosylated ligands	Not reported	Binds to several other ligands
Mannose 6-phosphate	7-8 x 10^{-6} M (1 mol/polypeptide)	7-8 x 10^{-6} M(2 mol/polypeptide)
PMP (Pentamannosyl Phosphate)	6 x 10^{-6} M	6 x 10^{-6} M
High mannose oligosaccharides with two phosphomonoesters or lysosomal enzymes	7-8 x 10^{-7} M	2 x 10^{-9} M
Methyl 6-phosphomannosyl diesters (from Dictyostelium discoideum)	No binding	Shows a weak Binding

* Note: Siva Kumar N. et al., ASCB conference Sanfransisco 2005; Siva Kumar N GLYCO-19 meeting, Crains, Australia, 2007.

1.5. Future perspective

A large number of a diversity of ligands (see table 1.2) bearing mannose 6-phosphate (M6P) signal that bind to CIMPR, suggests possible potential therapeutic applications. CIMPR could be targeted by synthetic M6P analogues carried by lysosomal proteins or drugs. CIMPR could activate several biological pathways either by acting at the cell membrane (TGFβ) or by internalizing extracellular ligands (glycoprotein D of the herpes virus, granzyme B and proliferin). Some structural features have been shown to be crucial for the binding of M6P to the receptor. The hydroxyl group at the 2-position of the pyranose ring must be axial to allow a strong binding to the CIMPR. Structural modifications of the M6P anomeric centre do not impair the binding to the CIMPR since fructose 1-phosphate (F1P) is recognized as easily as M6P (Tong et al., 1989; Distler et al., 1991). In order to improve the affinity for CIMPR, it would be interesting to use M6P analogues where the phosphate moiety has been replaced with other bio-isosteric groups. These analogues may improve both the affinity of the specific

recombinant enzyme for CIMPR and the stability of the M6P moiety in the blood circulation. Consequently, they could significantly enhance enzyme replacement therapy (ERT) efficacy and reduce the active doses of enzyme that is required for an efficient treatment. In a more recent review, these aspects have been discussed (Gary-Bobo et al., 2007)

Thus, proteins, vesicles, nano-particles or other polymers could be functionalized with M6P or its analogues in order to be used as carriers of bioactive molecules for the treatments of different diseases. An evident application of M6P derivatives is the transport of molecules to the lysosomes through the membrane CIMPR. An example in cancer therapy is explained in brief below.

1.5.1. Lysosomal targeting for cancer therapy

CIMPR has been reported to be a potential tumour suppressor in 70% of hepatocarcinomas (De Souza et al., 1995) and 15-30% of breast cancers (Hankins et al., 1996; Chappell et al., 1997; Oates et al., 1998). The gene was deleted in one allele and mutated at the other one in these cancers. Moreover, over-expression of CIMPR induced regression of tumours in mice and growth inhibition in cancer cells. These effects are probably due to the different functions of this receptor that indirectly controls cell growth : (i) internalization and degradation of various growth-promoting factors, such as IGF-II (Kiess et al., 1988), glycosylated Leukemia inhibitory factor (LIF), and other M6P-containing cytokines, such as the macrophage colony-stimulating factor (Blanchard et al., 1999); (ii) binding and uptake of granzyme B, an essential factor for T cell-mediated apoptosis (Motyka et al., 2000); (iii) regulation of the level of secreted lysosomal enzymes that are responsible for extracellular matrix degradation and tumour dissemination. Even though the CIMPR expression is decreased in some malignancies such as hepatocarcinoma, its overexpression in the majority of solid tumours, particularly in breast cancers (Berthe et al., 2003), indicate that this receptor could be considered as a mean to address cytotoxic drugs to lysosomes. The routing of cytotoxic drugs *via* CIMPR may induce the lysis of lysosomes and then cell death. The higher specificity of CIMPR targeting in cancer cells versus normal cells could be due to the higher expression of CIMPR and its higher affinity at slightly acidic pH (pH 6-6.5) as

found in the extracellular environment of solid tumors. This approach could be complementary to the classical chemotherapy. In fact, most anti-neoplastic drugs, such as anthracyclines (e.g.,doxorubicin) or *Vinca* alkaloids, are weakly basic molecules which target the neutral pH compartment of tumours, but become inactive in a more acidic environment (Kleeberger and Rottinger, 1993; Gerweck et al., 1999).

Other applications of the M6P analogues, which concern the competitive inhibition of different CIMPR extracellular ligands, have been already proposed depending on the patho-physiological condition. However, additional experiments on the physiological relevance of these unconventional M6P-based ligands and initial clinical trials should be performed before to consider the use of M6P analogues in the clinic. Finally, the M6P analogues could also be used to target drugs or nano-particles to lysosomes where they will be activated by the large panel of acid protease activities of these structures. This approach could be used in the case of photo-sensitive, or pH-sensitive, drugs for imaging or therapy in cancers that express high levels of CIMPR.

1.6. Scope of the present investigation

The laboratory where this work was undertaken has been working in the field of MPRs for the last 15 years, primarily towards understanding the evolution of these interesting proteins. The identification and extensive characterization of two distinct but related MPRs in different mammals (during the years 1986-1995) raised several interesting questions

i) Why nature made two MPRs that bind lysosomal enzymes?

Do the CDMPR and the CIMPR receptor have similar functions in lysosomal enzyme targeting and, if so, do they exhibit similar binding properties *in vivo?* Clearly, the CIMPR receptor is an important molecule required for transport of hydrolases to lysosomes. The lack of this protein in a few established cell lines results in secretion of a large fraction (60%) of newly synthesized lysosomal hydrolases, and this is prevented by expression of the receptor in the deficient cells (Kyle et al., 1988; Lobel et al., 1989). Interestingly, its targeting function is not significantly altered in cells that over express IGF-II (Braulke et al., 1991), consistent with the notion that it's two different ligands bind to different domains (Dahms et al., 1993, 1994).

ii) Among CDMPR and CIMPR which is more ancient in evolution?

Where in the evolution these two proteins have come into function, is there any other targeting system present which predates the existing targeting system? It was suggested that the two receptors may have a common ancestor, whereby the CIMPR receptor may arise from the more ancient M6P receptor by gene duplication and insertion during the course of evolution (Klier et al., 1991; Ludwig et al., 1991; Szebenyi and Rotwein, 1994; Dahms and Hancock, 2002). This is supported, in part, by evidence that the carbohydrate recognition function of the CDMPR receptor is conserved in both mammalian and non-mammalian species.

iii) Wherein evolution the MPR 300 protein has acquired its multifunctional nature?

To answer these questions, our laboratory has been involved in identifying these receptor proteins in the animal kingdom and eventually characterizing their biochemical and functional nature. Towards achieving our goal we have first purified and

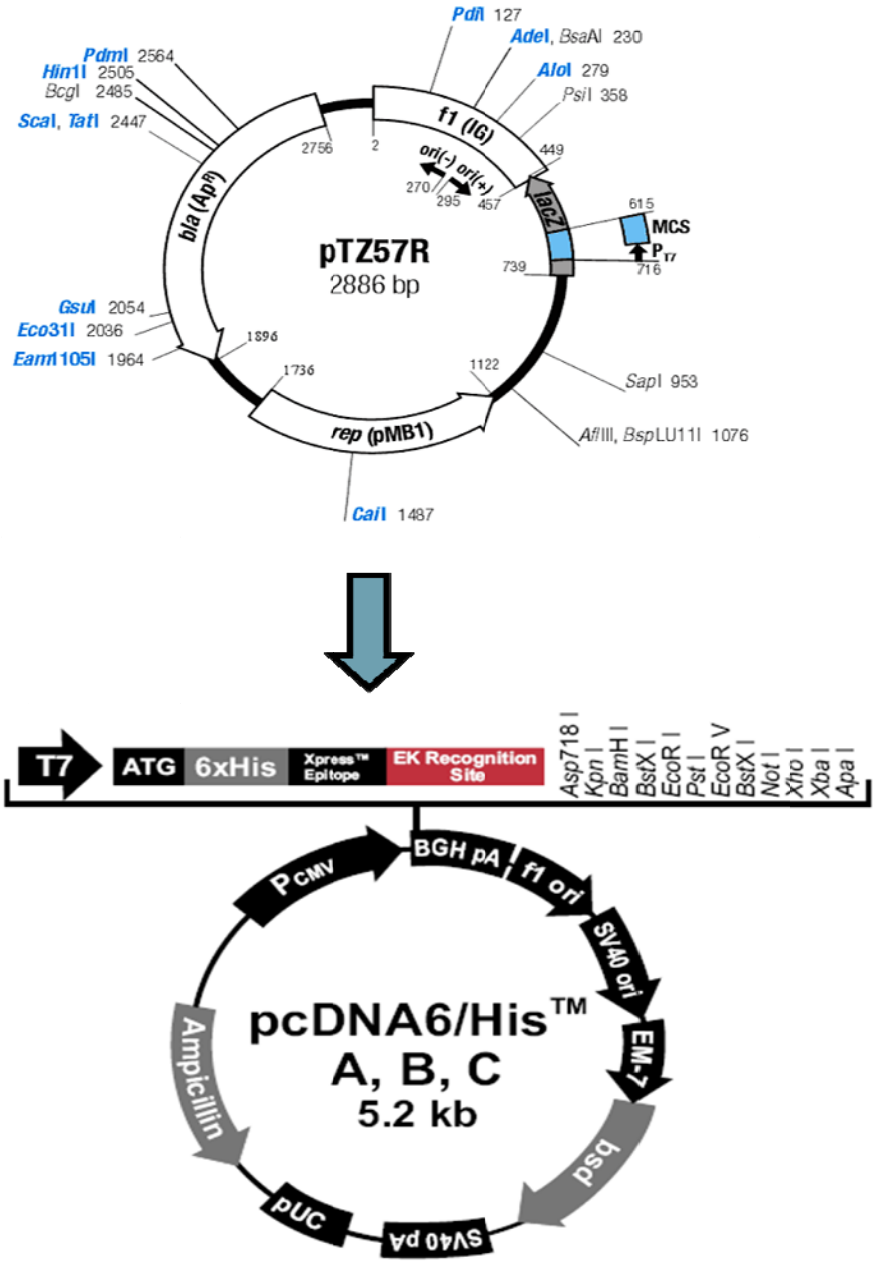
characterized MPRs from mammalian vertebrate goat and also in recent years cloned the full-length cDNA for MPR 46 and obtained partial clone for MPR 300 (Suresh et al., 2004). In the non-mammalian vertebrates both putative receptors have been identified in the chicken, reptiles, amphibians and fish (Matzner et al., 1996; Siva Kumar et al., 1997, 1999). We recently cloned and characterized the early vertebrate zebrafish MPR 46 gene (Suresh et al., 2005) and the fugu fish receptor genes (Siva Kumar et al., ASCB conference Sanfransisco, 2005; Siva Kumar GLYCO-19 meeting, Crains, Australia, 2007). Furthermore, the information available on the *xenopus* MPR 46 sequence also suggests that the MPR 46 is conserved in the vertebrates. In the invertebrate systems, so far the putative receptors have been affinity purified and biochemically characterized in the molluscs (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002) and more recently in the starfish (Sivaramakrishna and Siva Kumar, 2008). The study on star fish MPR 46 is the first report from our laboratory detailing its structure function relationship, which provided the evidence that the MPR 46 in the highly evolved invertebrate star fish is structurally related to the vertebrate receptor.

To establish that the CDMPR is conserved structurally and functionally among the different vertebrates, the present study was aimed at re-expressing the cDNAs for the CDMPR from goat and chicken in mouse embryonic fibroblast cells devoid of both the MPRs ($mpr^{(-/-)}$MEF cells). The expressed protein was characterized immunologically and its functional significance was established by studying its ability to bind and target cathepsin D (a lysosomal enzyme). The sorting function of MPR 300 in non-mammalian species is not as well characterized as the mammalian proteins, and there is no information on the invertebrate protein. To further gain knowledge on the functional significance of this receptor protein among the invertebrate species, the study was also extended to the invertebrates (molluscs). Mollusc cells, *Biomphalaria glabarata (Bg)* were used as model in this study. By metabolic labeling, blocking the MPR 300 present on cell surface with antibodies, its role in targeting lysosomal enzymes was unraveled. The binding specificity and binding parameters of the *Bg* cells MPR 300 was also determined using radioiodinated β-galactosidase (from bovine) and α-fucosidase (purified from *unio* tissue).

The results of our study support our strongly believed hypothesis that the MPR targeting system is an ancient pathway and it is possibly conserved from molluscs to mammals with respect to the MPR proteins. Though the mammalian homologues of both the receptors have been identified from molluscs to mammals, it still remains to be established if the lysosomal targeting system in the invertebrates; fish and birds is identical as in the mammals. Below the molluscs in the annelids and arthropods we could isolate only MPR 300 like proteins (Raju et al., 2001) and no MPR 46 protein was detected in these species. From *Drosophila melanogaster,* a Lysosomal Enzyme Receptor Protein (LERP) was identified that failed to bind the multivalent PM gel (Dennes et al., 2005), a characteristic feature exhibited by all the MPR proteins studied so far. Future studies should throw light on the possible targeting mechanisms for the lysosomal enzymes below the molluscs.

CHAPTER 2

Biochemical and functional characterization of cation dependent (Mr 46,000) goat mannose 6-phosphate receptor



2.1. INTRODUCTION

In order to understand the evolution of MPR proteins in the animal kingdom, we have previously identified the putative receptors from goat, chicken, reptiles, amphibians and fish (Suresh et al., 2004; Matzner et al., 1996; Siva Kumar et al., 1997; Siva Kumar et al., 1999). In the invertebrates, so far the receptor proteins have been affinity purified and biochemically characterized in molluscs (Sivaramakrishna and Siva Kumar, 2008), and in starfish (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002). We have also recently cloned and characterized the early vertebrate zebrafish MPR 46 gene (Suresh et al., 2005) and the fugu pufferfish (*Takifugu rubripus*) receptor genes (Siva Kumar, presented at ASCB conference Sanfransisco, 2005; GLYCO-19 Meeting, Glycocojugate J. 24, abstract No. 415, 2007). In the *Drosophila melanogaster, a* novel lysosomal enzyme recognition protein was discovered (Dennes et al., 2005). In prawn and earthworm only MPR 300 like protein could be detected (unpublished information). We have also characterized the goat receptors and cloned the genes for the same (Suresh et al., 2004). The extracytoplasmic domain, trans-membrane domain and the cytoplasmic tail are highly conserved. The potential residues essential for the M6P binding, cysteine residues that form the disulfide pairing in the amino terminal domain, dileucine motif DxxLL proposed to interact with sorting GGAs, endocytosis motif YRGV are conserved.

Earlier studies restricted to the purification, immunological characterization and cloning of goat MPR 46 protein (Suresh et al., 2004). The present study was aimed at understanding the functional significance of goat MPR 46 protein by expressing in MPR deficient cells. The full-length cDNA for the goat MPR 46 protein was expressed in $mpr^{(-/-)}$ MEF cells (that lack both the MPR proteins and therefore secrete the lysosomal enzymes into the medium) using a mammalian expression vector. The rationale would be if the goat receptor is expressed in these cells, it should restore the lysosomal enzyme targeting in these cells and also should be localized in these cells that can be detected by confocal microscopy. Furthermore, the receptor expressed should be able to be affinity purified. Employing this strategy, a detailed study was carried out to biochemically and functionally characterize the goat MPR 46 protein to understand its

structure-function relationship and to compare its properties with other known receptors. These are described in detail in the following chapter.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Cells: MPR-deficient mouse embryonic fibroblast cells ($mpr^{(-/-)}$ MEF cells) used in the present study were kindly provided by Prof. Dr. Regina Pohlmann, University of Muenster, Germany. The following antibodies were available in the laboratory. MSC1 antibody (an affinity purified human MPR 46 cytoplasmic tail antibody). 218 antiserum (an antiserum raised against the synthetic peptide corresponding to residues 218-232 in the cytoplasmic tail of the human MPR 46). Mouse cathepsin D antiserum raised in a rabbit was a generous gift from Prof. Dr. Regina Pohlmann. Lamp-1 monoclonal antibody was kindly provided by Prof. Dr. Stefan Hoening, University of Koeln, Germany.

2.2.2. Construction of expression vectors

The 840 bp full length goat MPR 46 cDNA clone that was originally cloned in pTZ57R vector (Suresh et al., 2004) was taken out and cloned into mammalian expression vector pcDNA.6/V5-His A (Invitrogen). Polymerase chain reaction (PCR) was performed using sense (5'-GG*A ATT CCC ACC ATG ATG TCC CCC CTC CAC-3') and anti-sense (5'-CCG C*TC GAG TCA CAT TGG TAA TAA GTG GTC-3') primers flanking *EcoRI and XhoI restriction sites. The amplified fragment, pcDNA.6/V5-His A (Invitrogen) vector were digested with EcoRI and XhoI restriction enzymes (MBI Fermentas). The digested fragment and linearized vector was excised and extracted using QIAEX II gel extraction kit. The vector and the insert were taken in 1:3 molar ratios for ligation reaction using T4 DNA Ligase (MBI Fermentas) and incubated at 22^{o}C for 3 h and then at 4^{o}C overnight. Ligated product was transformed into DH5α competent cells. Colony PCR was carried out to identify the colony harboring the gene of interest. The plasmid DNA was then isolated from one of the colony using Eppendorf fast plasmid kit and the presence of MPR 46 gene was confirmed by restriction

digestion. The sequence and reading frame was verified using automated DNA sequencing (Bioserve, India).

2.2.3. Cell culture and transfection

Mouse embryonic fibroblasts ($mpr^{(-/-)}$ MEF) deficient in MPR 46 and MPR 300, were grown in Dulbecco's minimal essential medium supplemented with 5 mM L-glutamine and 10% fetal calf serum (FCS). These cells secrete the newly synthesized lysosomal enzymes into the culture medium due to the lack of the putative receptors. Transfection by calcium precipitation method with pcDNA.6/ V5-His A vector construct in to these cells was done as below.

Twenty-four hours before transfection, rapidly healthy growing cells were trypsinized and reseeded them in growth medium into 6 cm petri dishes incubated overnight in a humidified incubator at 37ºC in 5% CO_2. 2x HBS (Hepes buffer saline, 280 mM NaCl, 50 mM Hepes acid, 1.5 mM Na_2HPO_4 (anhydrous) pH carefully adjusted to 7.05 with 5 M NaOH) is thawed and 250 µl is aliquoted into 1.5 ml centrifuge tube. In a separate 1.5 ml centrifuge tube 220 µl of 40 µg/ml DNA solution (if the amount of plasmid DNA is less, it is supplemented with salmon sperm DNA) was taken and to this 30 µl of 2 M $CaCl_2$ was added. Now the DNA/$CaCl_2$ mixture was added slowly (drop wise, while mixing) to 250 µl of 2x HBS over a period of about 1 min, and incubated at room temperature for 20 min, during which time a fine precipitate will be formed in the tube. To the petri plate, 5 ml of complete growth medium was added and then calcium precipitated DNA preparation was added at different areas. Later the plates were incubated overnight in a humidified incubator at 37ºC in 5% CO_2. To increase the transfection efficiency glycerol shock was generally given. The medium was removed completely and to this 2 ml of sterile filtered 10% glycerol in complete medium was added and left for 2-3 min at room temperature. To this 5 ml of PBS (10 mM sodium phosphate buffer pH 7.4, containing 150 mM NaCl) was added mixed gently and the glycerol/PBS/medium was removed completely. The plate was then washed twice with PBS; finally the cells were incubated for growth as usual in 5 ml of complete growth medium.

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

The goat MPR 46 and mock transfected cells were scraped with the help of a cell scraper, from the petri plate, 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 then lysed in 20 mM Tris-HCl buffer pH 7.4 containing 600 mM KCl, 20% glycerol, 1% Triton X-100, 1mM PMSF, 2 µg/ml leupeptin (Lysis buffer). The cell lysate was run on a 10% SDS-PAGE; proteins were transferred to nitrocellulose (NC) membrane and probed with MSC1 antibody to detect the expressed goat MPR 46 protein. To obtain the membrane extract from the cells expressing goat MPR 46, the cell pellet was collected as described above and was suspended in 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge, using a fixed angle 80Ti rotor at 161,280×g for 35 min. The pellet obtained at this step was dissolved in 50 mM imidazole-HCl buffer pH 7.0 containing 0.5% Triton X-100, sonicated as above, incubated for 20 min on ice, and recentrifuged as described above. The supernatant is designated as the total membrane extract and was used for the isolation of the expressed goat MPR 46 protein by Sepharose-divinlysulfone-phosphomannan gel (PM gel) chromatography.

2.2.5. Affinity chromatography

The fraction of membrane proteins obtained as mentioned above was applied on to PM gel at 4^{o}C pre-equilibrated with buffer containing 50 mM imidazole-HCl pH 7.0, 5 mM sodium β-glycerophosphate, 150 mM NaCl, 0.05% Triton X-100, 10 mM $MnCl_2$ (column buffer) (Siva Kumar et al., 1997). The flow rate of the column was maintained at 30 ml/h, and the gel was washed extensively with the same buffer. 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. The mannose 6-phosphate eluates were pooled and concentrated by acetone precipitation, aliquots analyzed by SDS-PAGE (under reducing and non-reducing conditions), western blotting and far western blotting. Divalent cation dependence of the purified receptor obtained above

was analyzed by performing the affinity chromatography as mentioned above by excluding the divalent metal ions in the column buffer.

2.2.6. SDS-PAGE and western blot analysis

Samples were analyzed by SDS-PAGE under reducing conditions as described (Laemmli, 1970). Gels were stained using the silver staining method (Blum et al., 1987). For immunological detection, aliquots of the purified proteins were electrophoresed, and the proteins were transferred to nitrocellulose membrane as described (Towbin et al., 1979). The membrane was placed in blocking solution containing 5% bovine serum albumin in phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST). The MPR 46 was detected using MSC1 antibody (2 µg/ml), and 218 antiserum (1:500 dilution). In case of 218 antiserum, alkaline phosphatase (ALP) conjugated goat anti-rabbit IgG (1:5000 dilution in PBST) was used as the secondary antibody. The blot was then developed with BCIP-NBT substrate in ALP buffer and the bands were visualized and photographed. Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:5000 dilution) was used as a secondary antibody for detecting the blot probed with MSC1 antibody. The blot was developed with enhanced chemiluminescence (ECL) reagent.

2.2.7. Immunofluorescence using confocal microscopy

$mpr^{(-/-)}$ MEF cells expressing goat MPR 46 were analyzed for the MPR 46 protein by confocal microscopy using MSC1 antibody. After transfection, 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 and blocked with 5% BSA in PBS for 30 min at room temperature. The cells were then incubated with MSC1 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, incubated with fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (molecular probes, Invitrogen) for 1 h at room temperature. Finally the cells were washed extensively and observed under confocal microscope. The $mpr^{(-/-)}$ MEF cells expressing goat MPR 46 were analyzed for the cathepsin D retention using cathepsin D antiserum

by immunofluorescence. Lysosomes were labeled by Lamp-1 antibody. After fixation and permeabilization the cells were incubated with cathepsin D antiserum (1:250 dilution) and Lamp-1 antibody (1:100 dilution) in 5% BSA, the cells were then incubated with anti-rabbit IgG-Alexa fluor 594 followed by anti-mouse IgG-FITC (Bangalore Genei).

2.2.8. Far-western blotting and coimmunoprecipitation

The medium collected from the cultured $mpr^{(-/-)}$ MEF cells was precipitated by addition of 50% ammonium sulfate and the protein pelleted by centrifugation in a Biofuge stratos centrifuge, using a fixed angle (8 × 50 ml) rotor at 17,226×g for 20 min. It was dissolved in 10 mM Tris-HCl pH 7.4, containing 150 mM NaCl (TBS) and dialyzed against TBS. Aliquot of the dialyzed sample was then separated on 10% SDS-PAGE and analyzed by western blot (Towbin et al., 1979) and far-western blot as described (Ikushima et al., 2000). The western blot and far-western blot analysis was carried out using the anti-cathepsin D antiserum and MSC1 antibody, respectively. In both cases, the detection was carried out by alkaline phosphatase conjugated secondary antibodies. For coimmunoprecipitation of cathepsin D and goat MPR 46, 100 µl each of transfected cell lysate was taken into three different tubes. To each tube 900 µl of 20 mM Hepes pH 7.4 buffer containing 300 mM NaCl, 4 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM PMSF, 2 µg/ml leupeptin was added. Tube 1 received 1 µl of preimmune serum. Tube 2, was preincubated for 3 h with 5 mM M6P, while tube 3 did not receive M6P. 1 µl each of cathepsin D antiserum was added to tube 2 and 3. The tubes were kept for rotation overnight at 4^{o}C. To each tube, 20 µl of protein A-agarose (Bangalore Genei) was added and rotated for 4 h at 4^{o}C. The reaction tubes were then briefly centrifuged in a table top centrifuge, washed with phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST) 6-7 times. The pellet was cooked in 2X SDS-PAGE sample buffer and loaded on a 10% SDS- PAGE under reducing conditions. The proteins after separation were transferred to nitrocellulose membrane. The membrane was probed with MSC1 antibody, processed as described above and the protein bands were detected using ECL reagent.

2.3. RESULTS

2.3.1. Construction and analysis of expression vectors for the presence of MPR 46

Amplification of the goat MPR 46 cDNA was carried out using the primers as described under materials and methods. The amplified product corresponding to 840 bp in length (Fig 2.4.1A lane 2) was cloned into the mammalian expression vector. The presence of insert in the expression vector was confirmed by subjecting the vector to EcoRI and XhoI digestion and analysis of the products on an agarose gel (Fig 2.4.1B lane 4), sequence and reading frame was confirmed by automated DNA sequencing. Transfections were made in $mpr^{(-/-)}$ MEF cells that missort up to 80% of soluble lysosomal enzymes to the medium due to the lack of MPR 46 and MPR 300 proteins. The deficiency results in abnormal size and number of intracellular storage lysosomes that cannot degrade ingested materials. In these cells, the missorting of lysosomal enzymes can be rescued by the expression of a receptor protein that re-establishes functional sorting (Kasper et al., 1996).

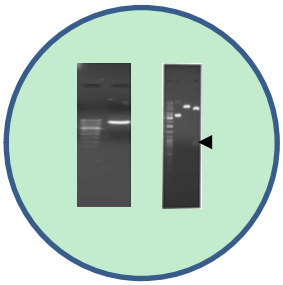
2.3.2. Expression of the protein

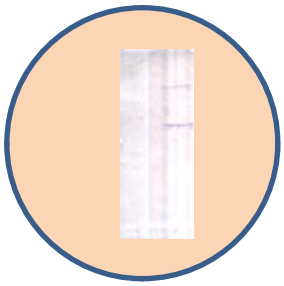
The cell lysate from the $mpr^{(-/-)}$ MEF cells that were transfected with the expression vector containing goat MPR 46, and mock transfected cells were probed with MSC1 antibody under the same conditions. The blot was developed with BCIP/NBT substrate, specific band corresponding to the monomeric and dimeric forms of the receptor was observed in the transfected cell lysate (Fig 2.4.2 lane 2) but not in the mock transfected cell lysate (Fig 2.4.2 lane 1). Membrane fractions from the transfected cells were prepared and subjected to affinity chromatography on PM gel. Bound protein was specifically eluted with 5 mM M6P, and analyzed on SDS-PAGE (Fig 2.4.3A). From the results it is evident that the putative receptor was found in the mannose 6-phosphate eluate and exhibited a molecular mass similar to the purified MPR 46 receptor from goat liver tissue. In addition to the monomeric form, a dimeric form of MPR 46 has been observed (Fig 2.4.3A, lane 4). The oligomeric nature was observed both under non-reducing (Fig 2.4.3B lane 2) and reducing conditions (Fig 2.4.3B lane 3). The authenticity of the receptor was further confirmed by immunoblotting using an

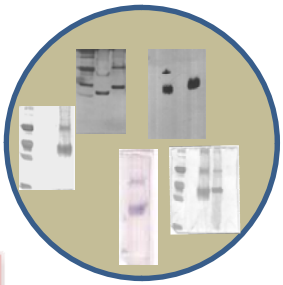
MSC1 antibody wherein the purified goat liver MPR 46 protein served as a positive control (Fig 2.4.3C lane 1 and 2) and 218 antiserum respectively (Fig 2.4.3D). In the absence of divalent metal ions, the receptor was completely recovered in the unbound fraction suggesting that PM gel binding depends on divalent metal ions (Fig 2.4.4 LANE 4).

2.3.3. Localization, interaction of the lysosomal protease cathepsin D with expressed goat MPR 46 protein

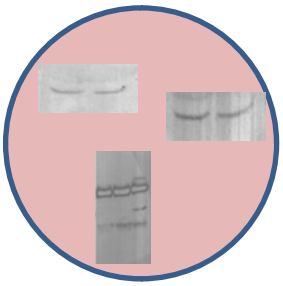
The distribution of the expressed goat MPR 46 protein was detected by immunofluorescence using MSC1 and Lamp-1 antibody (Fig 2.4.5). The sorting function of the lysosomal protease cathepsin D to lysosomes in the goat MPR 46 transfected $mpr^{(-/-)}$ MEF cells was demonstrated by confocal microscopy using the cathepsin D antiserum (Fig 2.4.6F). The lysosomes in the same cells were visualized using Lamp-1 antibody (Fig 2.4.6E). Merged image (Fig 2.4.6G) of both the images (Fig 2.4.6E and 2.3.6F) showed extensive colocalization of cathepsin D and Lamp-1. The goat MPR 46 expressing MEF cells clearly improved intracellular steady state concentrations of cathepsin D as shown in Fig 2.4.6F, which was missorted to the medium by the MEF cells that were deficient for both the MPRs as shown in Fig 2.4.6B. The presence of cathepsin D in $mpr^{(-/-)}$ MEF secreted medium was detected by immunoblotting as shown in Fig 2.4.7A. The interaction of expressed goat MPR 46 protein with cathepsin D was confirmed by far-western blotting as shown in Fig 2.4.7B. The specific interaction was further confirmed by coimmunoprecipitation experiment with cathepsin D antiserum. The preimmune serum was used as a control (Fig 2.4.7C lane 1). Mannose 6-phosphate dependent binding was also analyzed (Fig 2.4.7C lane 2). The specific identification of the goat MPR 46 monomeric and dimeric forms indicates the interaction of goat MPR 46 protein with the cathepsin D (Fig 2.4.7C lane 3).

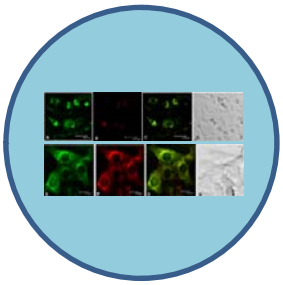






FIGURES 2.4





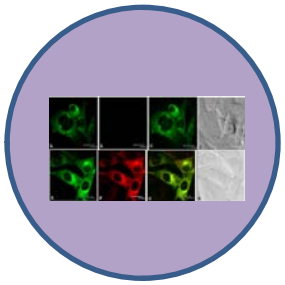


Figure 2.4.1: **Cloning of goat MPR 46 gene into pcDNA.6/V5-His A.** 0.8% agarose gel electrophoresis. **(A) Amplification of goat MPR 46,** lane 1, 100 bp ladder, lane 2, specific amplification of 840 bp. **(B) Restriction analysis**, lane 1, mixed DNA ladder, (10,000 to 100 bp) lane 2, undigested goat 46 in pcDNA.6/V5-His A, lane 3, single digested goat 46 in pcDNA.6/V5-His A (with EcoRI), and lane 4, double digested goat 46 in pcDNA.6/V5-His A (with EcoRI and XhoI).

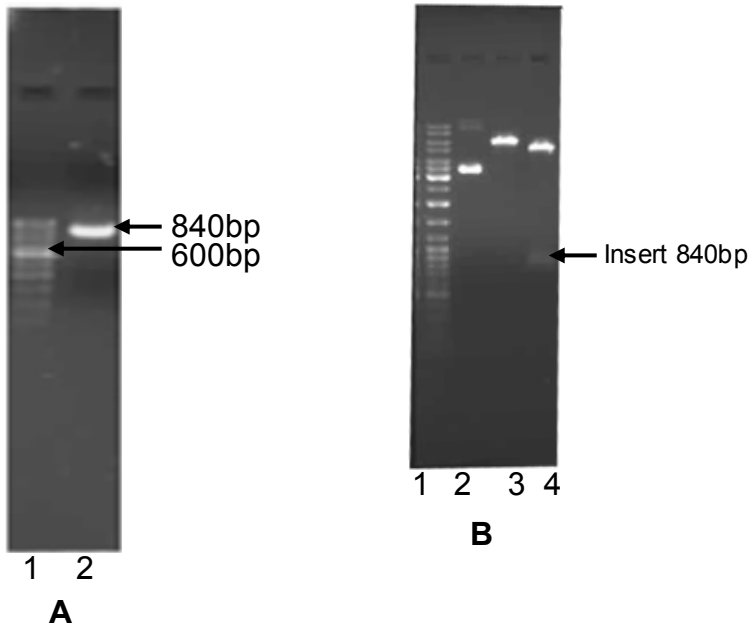


Figure 2.4.1

Figure 2.4.2: **Western blot analysis of the mock transfected and transfected cell lysates**. Lane 1, mock transfected cell lysate, lane 2, transfected cell lysate, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with MSC1 antibody (2 µg/ml). Both the monomeric (M) and dimeric (D) forms were observed.

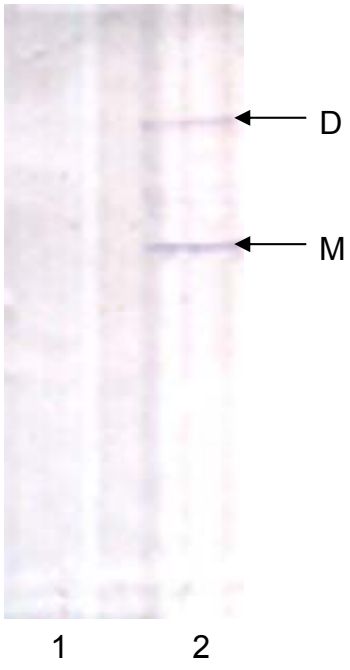


Figure 2.4.2

Figure 2.4.3: **(A) Purification of expressed goat 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) Analysis of the purified protein under reducing and non-reducing conditions**, lane 1, Molecular weight markers, lane 2, mannose 6-phosphate eluate of goat 46 from the PM Gel under non-reducing conditions, lane 3, mannose-6-phosphate eluate of goat 46 from the PM Gel under reducing conditions (+ DTT). (**C) Western blot analysis of the mannose 6-phosphate eluate of goat 46 from the PM Gel, probed with MSC1 antibody**. Lane 1, Mannose 6-phosphate eluate showing monomeric (M) and dimeric (D) forms. Lane 2, goat MPR 46 purified from liver tissue serving as a positive control. **(D) Western blot analysis of the mannose-6-phosphate eluate of goat 46 from the PM Gel, Probed with 218 peptide specific antiserum.**

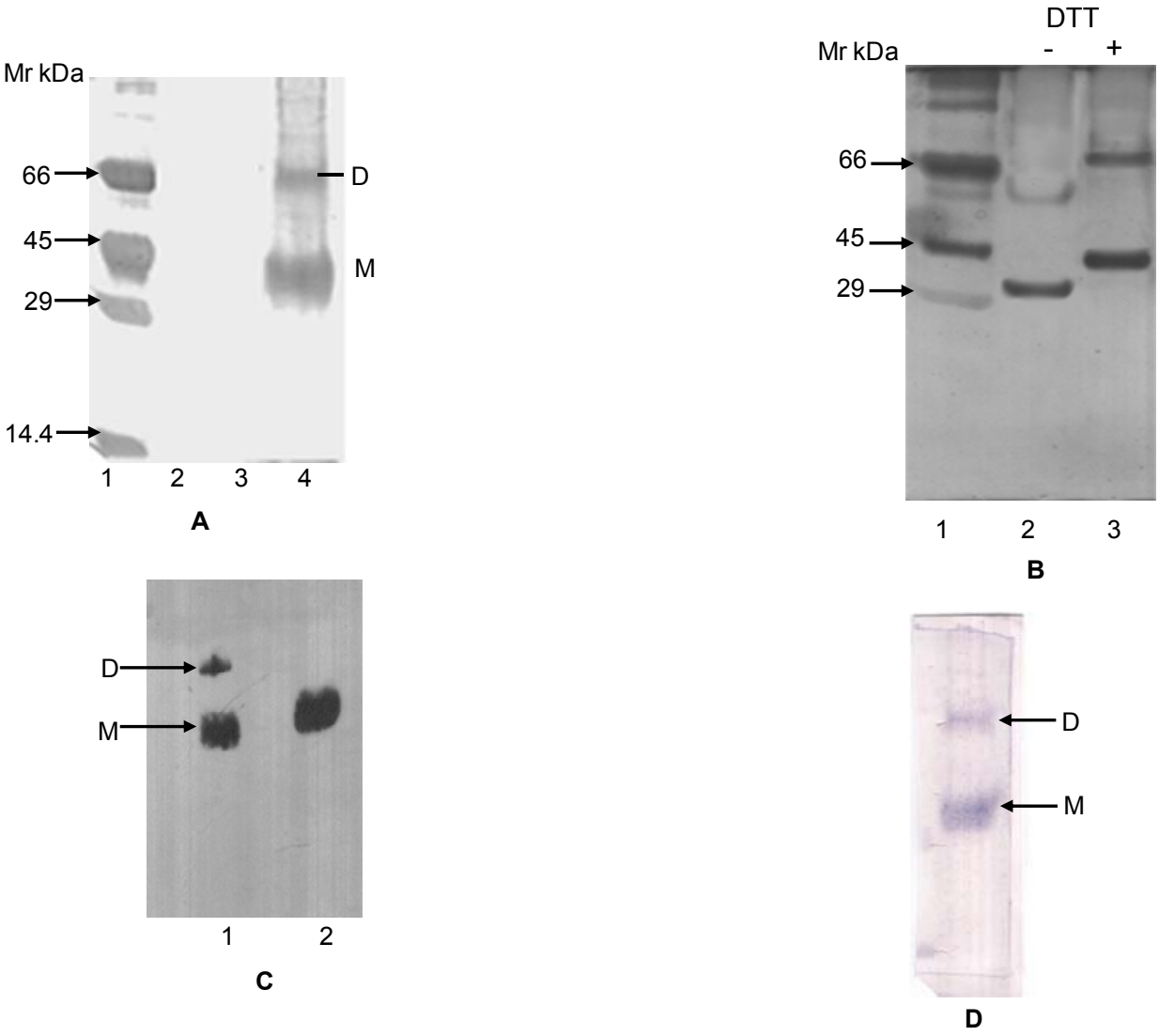


Figure 2.4.3

Figure 2.4.4: **Requirement for divalent cations by goat MPR 46 protein to bind PM gel**. An aliquot of the purified, acetone precipitated sample was loaded on a 1 ml PM gel column, one equilibrated with the buffer containing divalent cations and the other without cations. The gel after washing, eluted with 5 mM M6P in respective column buffers. Both the unbound and eluted fractions were loaded on SDS-PAGE. Lane 1, molecular weight marker, lanes 2 and 3 are unbound and eluate in presence of divalent cations. Lanes 4 and 5 are unbound and eluate in the absence of divalent cations.

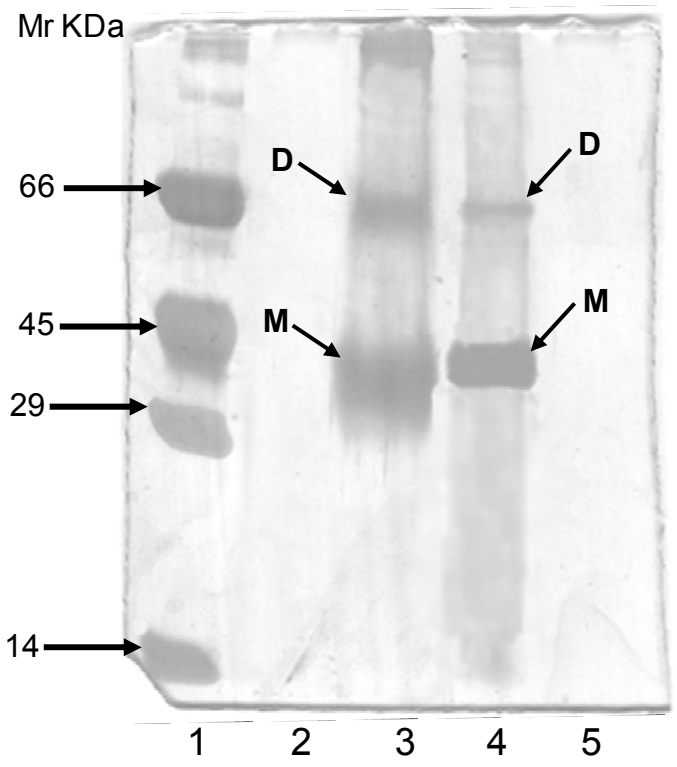


Figure 2.4.4

Figure 2.4.5: **Immunofluorescence of the expressed protein.** Immunofluorescence with Lamp-1 antibody in $mpr^{(-/-)}$ MEF cells (A) and in goat MPR 46 expressing cells (E). Immunofluorescence using MSC1 antibody in $mpr^{(-/-)}$ MEF cells (B) and in goat MPR 46 expressing cells (F). (C) merged image of (A) and (B), (G) merged image of (E) and (F). (D) and (H) are the corresponding transmission images. Bar in the top panel is 14.65 μm and the bottom panel is 12.24 μm.

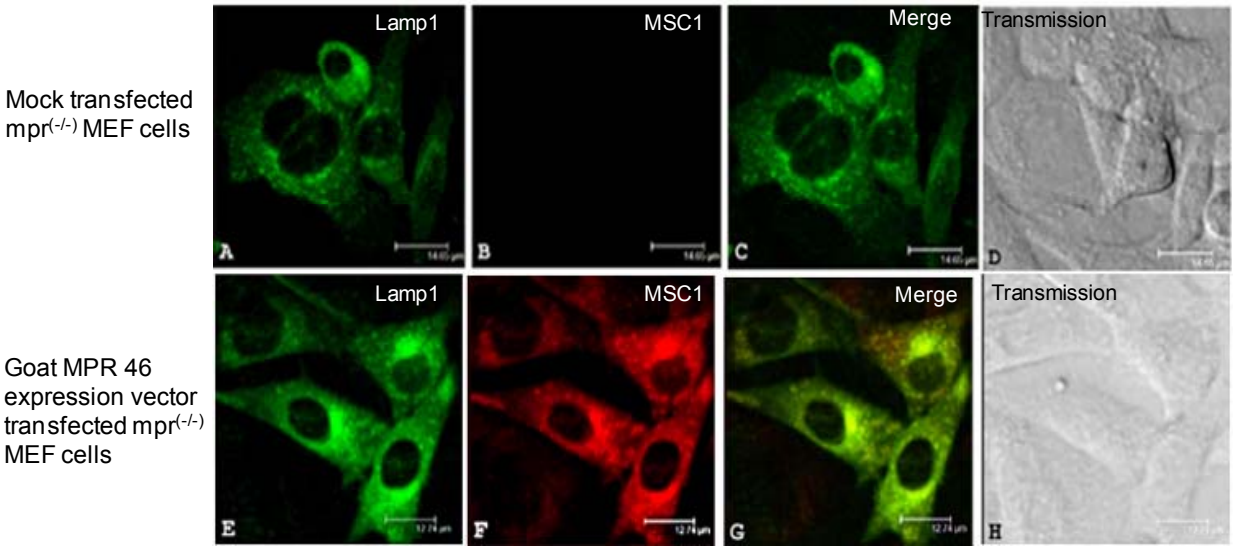

Mock transfected mpr(-/-) MEF cells
Goat MPR 46 expression vector transfected mpr(-/-) MEF cells
Lamp1
MSC1
Merge
Transmission
A
B
C
D
E
F
G
H

Figure 2.4.5

Figure 2.4.6: **Sub cellular distribution and localization of cathepsin D in goat MPR 46 expressing cells visualized using confocal microscopy**. Immunofluorescence using Lamp-1 antibody in $mpr^{(-/-)}$ MEF cells (A) and in goat MPR 46 expressing cells (E). Immunofluorescence using cathepsin D antiserum in $mpr^{(-/-)}$ MEF cells (B) and in goat MPR 46 expressing cells (F). (C) merged image of (A) and (B), (G) merged image of (E) and (F). (D) and (H) are the corresponding transmission images. Extensive co-localization of cathepsin D and Lamp-1 is observed in cells expressing goat MPR 46. Bar in the top panel is 43.5 μm and the bottom panel is 12.34 μm.

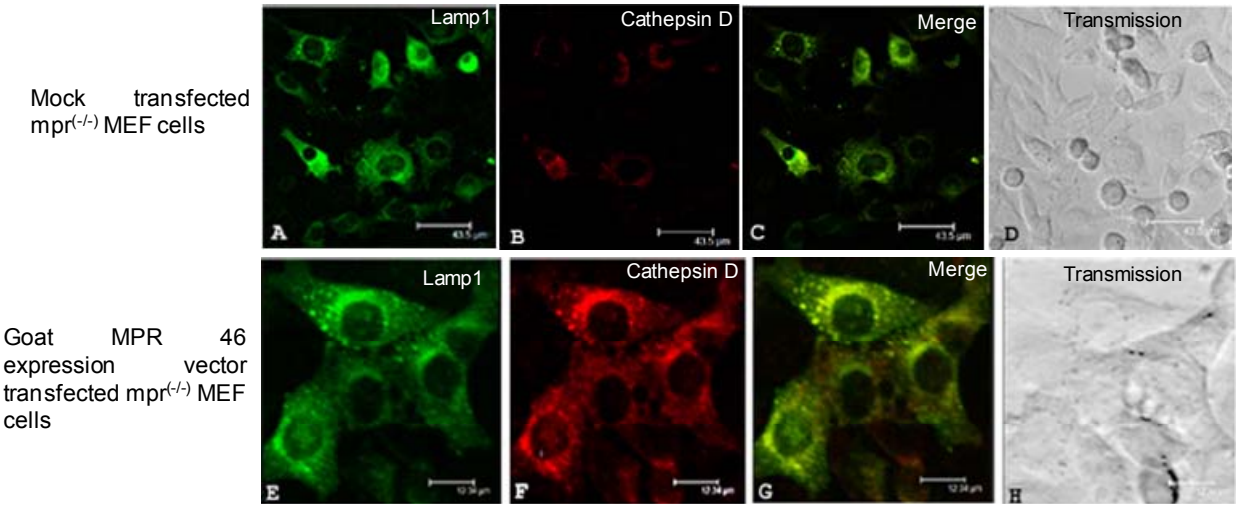

Mock transfected mpr(-/-) MEF cells
Goat MPR 46 expression vector transfected mpr(-/-) MEF cells
Lamp1
Cathepsin D
Merge
Transmission
A
B
C
D
E
F
G
H

Figure 2.4.6

Figure 2.4.7: **(A) Western blot of mpr$^{(-/-)}$ MEF cells secreted medium probed with cathepsin D antiserum**. **(B) Far-Western blotting**, mpr$^{(-/-)}$ MEF cells secreted medium was separated on 10% SDS-PAGE, transferred onto NC membrane and incubated with purified expressed goat MPR 46. Bound MPR 46 to cathepsin D was detected by using MSC1 Ab. Lanes 1 and 2 are duplicates of the proteins obtained from the mpr$^{(-/-)}$ MEF cells secreted medium. **(C) co-immunoprecipitation of the cell lysates prepared from mpr$^{(-/-)}$ MEF cells transfected with goat MPR 46 using cathepsin D antiserum** (details given under methods). 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.(★) Indicated in the figure shows the non-specific signal due to the IgG heavy chain and light chain. M and D represent the dimeric and monomeric forms of the receptor protein.

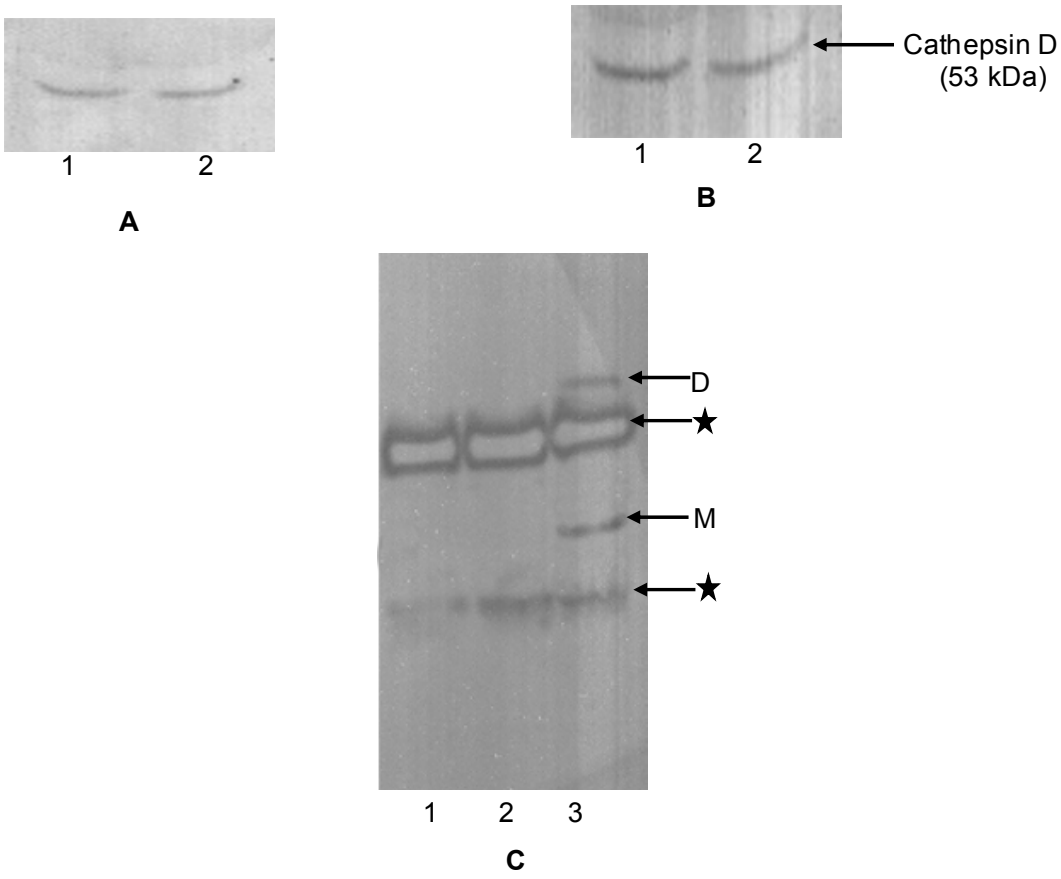


Figure 2.4.7

Table 2.1. *Similarity of the various regions of the goat MPR 46 protein with other known vertebrate MPR proteins*

Species	*Size of Mature protein (kDa)*	*cDNA clone size*	transmembrane domain	Ligand binding region sequences and / *conserved residues in cytoplasmic tail (italics)*	Potential glycol-sylation sites / *conservation of cysteins (italics)*	*Size of RNA trans-cript (kb)*
HUM	46	0.837	ILLVTFASLVAVYVVGGFLY	DNHCGKEQ**RR**AV ***YRGV*** / *DXXLL*	conserved/ *yes*	2.3
BOV	46	0.840	ILLVTFASLVAVYVVGGFLY	DNHCGREQ**RR**AV ***YRGV*** / *DXXLL*	conserved/ *yes*	2.3
MOU	46	0.837	ILLVTFASLVAVYVVGGFLY	DNHCGKEQ**RR**AV ***YRGV*** / *DXXLL*	conserved/ *yes*	2.3
GOAT	46	**0.840**	ILLVTFASLVAVYVVGGFLY	DNHCGREQ**RR**AV ***YRGV*** / *DXXLL*	conserved/ *yes*	2.3
CHI	46	0.84	ILLVTFASLVAVYVVGGFLY	GRHCSGEK**RR**AV ***YRGV*** / *DXXLL*	conserved *yes*	3.3
XEN	46	partial	ILLVTFASLVAVYVVGGFLY	DTHCNNEA**R**KAM ***YRGV*** / *DXXLL*	conserved *yes*	not known
KIL	46	partial	ILLVTFASLVAVYVVGGFLY	DSHCQKEA**R**KAII ***YRGV*** / *DXXLL*	conserved *yes*	not known
ZEB	38	0.84	ILLVTFASLVAVYVVGGFLY	DSHCSSEE**R**KAM ***YRGV*** / *DXXLL*	conserved *yes*	not known

HUM, human, BOV, bovine, MOU, mouse, CHI, chicken, XEN, *Xenopus*, KIL, killi fish, ZEB, zebrafish, **R**: Conserved arginine residue in MPR 46 responsible for ligand binding. Conserved sequences in the cytoplasmic tail: *bold italics:* tyrosine dependent endocytosis motif: ***YRGV,*** Conserved acidic dileucine motif in italics.

2.5. DISCUSSION

Lysosomal biogenesis is important in many physiological and pathological processes. In mammals it has been shown that two distinct but homologous receptors designated as the MPR 300 and 46, mediate the transport of the lysosomal enzymes to lysosomes. The role of MPRs in the biogenesis of lysosomes is illustrated by the excessive secretion and intracellular deficiency of lysosomal proteins in cells from patients with I-cell disease, which lack the phosphotransferase required for the biosynthesis of the M6P containing recognition marker on lysosomal enzymes (Kornfeld and Sly, 1995). The physiological role of the MPR 46 for the transport of lysosomal enzymes has remained an enigma mainly because mammalian cells express simultaneously two MPRs. Only a few tumour-derived cell lines that have lost the expression of the MPR 300 are an exception to this rule (Gabel et al., 1983; Mainferme et al., 1985). In these cells the greater part of newly synthesized lysosomal enzymes are secreted. Secretion is even further enhanced in these cells when the endogenous MPR 46 is functionally inactivated by blocking with antibodies, and secretion can be reduced, in part, by over expression of MPR 46 (Stein et al., 1987b; Watanabe et al., 1990; Ma et al., 1991). From these observations it was concluded that MPR 46 participates in the transport of newly synthesized lysosomal enzymes to lysosomes. Analysis of these cell lines deficient in MPR obtained from mice with a targeted disruption of the respective MPR gene (Köster et al., 1993; Ludwig et al., 1993; Wang et al., 1994; Pohlmann et al., 1995) or from tumors (Gabel et al., 1983) showed that each of the two MPRs contributes to the targeting of newly synthesized lysosomal proteins, and that the complements of lysosomal enzymes that are transported by either receptor are distinct but largely overlapping. Köster et al. (1993) clearly demonstrated that the complete loss of MPR 46 is associated with a misrouting of mannose 6-phosphate containing polypeptides out of the cell. This strongly argues for an essential function of MPR 46 in the targeting of newly synthesized lysosomal enzymes and that this function cannot be compensated for by the endogenous MPR 300.

Mouse embryonic fibroblasts that are deficient in the two mannose 6-phosphate receptors (MPRs) MPR 46 and MPR 300 missort the majority (≥85 %) of soluble lysosomal proteins into the medium. These cells are used by various investigators to

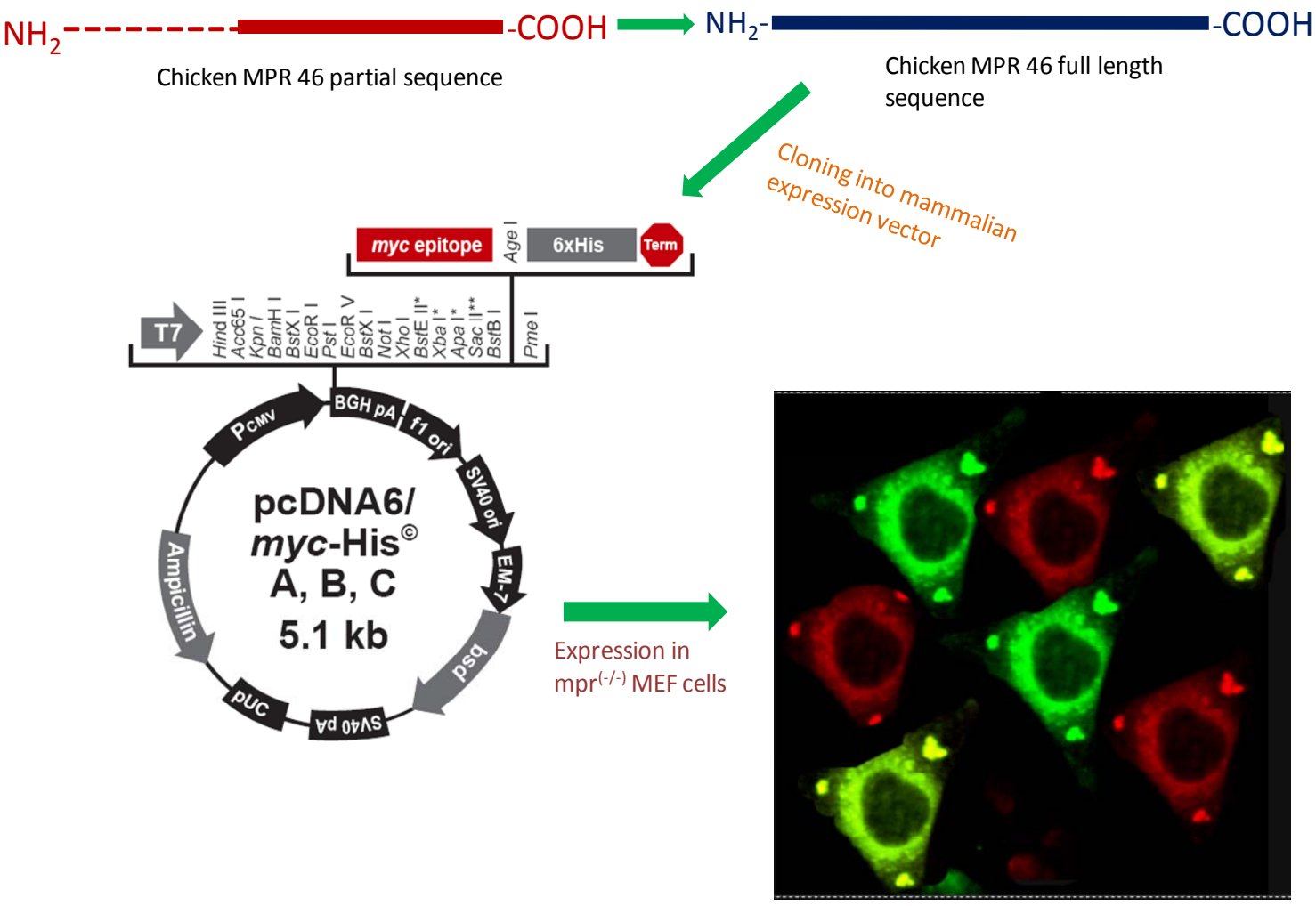
study the role of MPR 300 and MPR 46 proteins from human (Kasper et al., 1996; Denzer et al., 1997) and bovine sources (Marron-Terada et al., 1998). These cells were used in the present study to characterize the goat MPR 46. Our laboratory first purified and characterized the goat MPR proteins and has also developed an ELISA method to quantify the receptors (Suresh et al., 2003). Furthermore to understand the structural similarities of the goat receptors to that of other known mammalian and non-mammalian receptors, we have also obtained a full-length clone for the goat MPR 46 and a partial cDNA clone for the goat MPR 300 protein (Suresh et al., 2004). Results of these experiments revealed that the goat receptor is homologous to the other known vertebrate receptors particularly the bovine receptor. Table 2.1 summarizes the homology of the goat receptor to the other known vertebrate receptors. The homology of the goat receptor is significant particularly with respect to the location of the arginine residue that is critical to the lysosomal enzyme binding, the sugar binding pocket as well as the metal binding regions. It is therefore of interest to express the goat MPR 46 in $mpr^{(-/-)}$ MEF cells to compare its properties to those reported previously for the bovine and human receptors and study its intracellular sorting of lysosomal enzymes and hence the present study was carried out to gain further insight into the biological function of the goat MPR 46 receptor. It is well established that the mammalian MPR 46 protein shows an absolute requirement for divalent metal ions to bind on PM gel. Consistent with the earlier reports (Hoflack and Kornfeld, 1985; Waheed and von Figura, 1990) we also found that the expressed goat MPR 46 protein binds to the PM gel only in the presence of divalent metal ions. The expressed protein in SDS-PAGE analysis exhibited a molecular mass of ~46 kDa, in addition to this monomeric form; the dimeric form of the receptor could also be detected. Both these forms were also recognized by the MSC1 antibody as well as the 218 antiserum used, which are known to recognize the oligomeric forms of the receptor (Siva Kumar et al., 1991). The oligomeric nature was also supported by the results obtained in the SDS-PAGE analysis of the receptor in the presence and absence of reducing agent. The expressed protein in the cells was observed by immunofluorescence using confocal microscopy. In a recent study we have demonstrated the expression of the Zebra fish MPR 46 protein in $mpr^{(-/-)}$ MEF cells (Suresh et al., 2005). With these studies it has been shown that the

expressed Zebra fish MPR 46 sorts the cathepsin D. In order to verify that the expressed goat receptor protein is capable of binding to the cathepsin D, an antiserum to cathepsin D was used in immunofluorescence studies. From our results it is clear that the expressed goat MPR 46 protein is capable of binding and sorting newly synthesized lysosomal enzymes was suggestive by the increase in the localization of the cathepsin D in the expressing cells compared to the $mpr^{(-/-)}$ MEF cells (compare panel 2.4.6B and 6F). This was also supported by the far-western blot analysis and coimmunoprecipitation, which confirmed the specific interaction of goat MPR 46 to the cathepsin D which is dependent upon M6P.

In summary our results clearly demonstrate the following (i) the full length goat MPR 46 cDNA can be expressed in $mpr^{(-/-)}$ MEF cells (ii) the expressed protein can be affinity purified on a PM gel in the presence of divalent metal ions and exhibits typical biochemical and immunological characteristics similar to the well characterized mammalian receptors as evidenced by our results (iii) the expressed protein was observed in confocal microscopy (iv) further evidence that the expressed protein is sorting lysosomal enzymes came from immunofluorescence studies using the cathepsin D (a known lysosomal enzyme) antiserum. In view of the structural similarities the goat receptor exhibits with other well characterized MPR 46 proteins, and the results of the present study establishing the functional property of the goat receptor viz; PM gel binding, cation dependence to bind on PM gel, as well as specific sorting of cathepsin D provide evidence that the goat protein studied here is the putative lysosomal enzyme targeting receptor.

CHAPTER 3

Molecular cloning, expression and functional characterization of the chicken cation dependent mannose 6-phosphate receptor protein



3.1. INTRODUCTION

Among the two MPRs, only MPR 300 has been shown to be a multifunctional protein capable of binding and endocytosis of a variety of ligands that are involved in embryogenesis, differentiation and immunity (Dahms and Hancock, 2002). On the other hand, MPR 46 functions in transport of internal lysosomal enzymes and does not endocytose extracellular ligands. However it is still not clear why nature developed two receptors and whether different vertebrate animals use the same mechanism to transport their newly synthesized lysosomal enzymes.

In view of this special characteristics exhibited by the mammalian MPR 300 receptor. Particularly its ability to bind human IGF-II in addition to mannose 6-phosphate containing ligands, it is important to understand the structure–function of these receptors and their evolution. Zhou et al. (1995) cloned chicken MPR 300 gene, sequenced and showed that it mediates transport of lysosomal enzyme β-glucuronidase. However, the chicken receptor differed in its sequence in the 11th domain compared to the mammalian protein, and therefore it was suggested that the chicken receptor binds human IGF-II with low affinity and it was suggested possibly the IGF-II binding ability of the receptor is a late event in evolution (Zhou et al., 1995). Only recently this protein has also been shown to bind human IGF-II and is thus an endocytic and multifunctional receptor like the mammalian protein (Suresh et al., 2006). Matzner et al., 1996 showed that in chicken both MPR proteins exhibit distinct expression patterns. Only a partial cDNA clone for the chicken MPR 46 is known which suggested homology to the mammalian proteins and its localization in the developing chicken was studied, though its functions have been poorly characterized.

Our recent detailed studies on the goat (mammalian) MPR 46 revealed that the protein is structurally and functionally similar to several mammalian proteins and also to the recently characterized zebrafish (early vertebrate) receptor. Furthermore, the information available on the *Xenopus* MPR 46 sequence also suggests that the MPR 46 is conserved in the vertebrates. We also found that the receptor levels vary in different tissues of goat and chicken (Suresh et al., 2003). Since chicken forms the first non-mammalian vertebrate in the evolutionary tree, it becomes necessary to obtain the complete sequence information and understand the function of MPR 46 from this

species. To study this we went onto obtain the complete sequence information of the chicken receptor and studied its function to gain insights into the evolution of the vertebrate MPR 46. Therefore the present study was carried out with the following objectives, (i) to prepare a full length cDNA for the chicken receptor and to make a structural comparison of the sequences to other known vertebrate receptors, (ii) to re-express the MPR 46 cDNA in $mpr^{(-/-)}$ MEF cells to study its function, and (iii) to analyze the receptor ability to bind phosphomannan (PM gel) and to a lysosomal enzyme cathepsin D. The results presented here is the first report on the complete cDNA cloning, sequencing and functional characterization of the chicken MPR 46.

3.2. MATERIALS AND METHODS

3.2.1. Materials

MPR-deficient mouse embryonic fibroblast cells ($mpr^{(-/-)}$ MEF cells) used in the present study are devoid of both the receptors and were kindly provided by Prof. Dr. Regina Pohlmann, University of Muenster, Germany. The media for cell culture, primers and the chromogenic substrates used for enzyme assays were purchased from Sigma. Blasticidin S was purchased from Invitrogen. The antibodies used in this study were same as described for the goat MPR 46 protein in the proceeding chapter.

3.2.2. Isolation of total RNA from chicken liver

Total RNA was isolated from about 30 mg of chicken liver tissue using RNeasy kit (Qiagen). Purity and integrity of the RNA was checked on 1% agarose gel under denaturing conditions.

3.2.3. Amplification and cloning into expression vector

From the RNA obtained above, 5 µg was taken and reverse transcription (RT) reaction was done with oligo dT following instructions of the cDNA synthesis kit (MBI Fermentas). 2 µl from the RT reaction was used as template in the next polymerase chain reaction (PCR) step. PCR was performed using the following primers with the restriction sites shown in there. Sense primer: 5'-CGG*AATTCATGACGGTGCAC-3' (*

EcoRI site). Antisense primer 5'- CCGC*TCGAGTCACATCGGTAGCAA -3' (* Xho1 site). The amplified fragment and pcDNA.6/V5-His A vector (Invitrogen) were digested with EcoRI and XhoI restriction enzymes (MBI Fermentas). The double digested insert and vector were run on agarose gel, bands excised and extracted using the Qiagen gel extraction kit. The insert was then ligated into the vector using ligation kit (MBI Fermentas). The ligated product was transformed into DH5α competent cells. Colonies so obtained were then tested for the presence of the insert by restriction digestion, the sequence and reading frame was verified using automated DNA sequencing (Bioserve, and also by Sigma, India).

3.2.4. Transfection of cell lines

Immortalized mouse embryonic fibroblasts ($mpr^{(-/-)}$ MEF) deficient in MPR 46 and MPR 300 were transfected with 20 µg of pcDNA.6/V5-His A containing the chicken MPR 46 gene by calcium precipitation method as described (page 34, section 2.2.3).

3.2.5. Selection of stable cell lines

Stable cell lines expressing the chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) were selected in Blasticidin S antibiotic containing medium with a final concentration of 15 µg/ml. The selection medium was changed for every 3 days. About 20 clones were selected and quantitated for the expression level of MRP 46 protein by RT-PCR taking equal amounts total RNA from different clones, chicken embryonic fibroblast (CEF) cells, mouse embryonic fibroblast (MEF) cells and mock transfected $mpr^{(-/-)}$ MEF cells. The clone expressing the physiological level of MPR 46 protein was expanded further.

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

The cell lysate preparation and membrane protein extraction from chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) and mock transfected (vector alone) $mpr^{(-/-)}$ MEF cells was carried out as described (page 35, section 2.2.4).

3.2.7. Purification of chicken MPR 46 protein

The expressed chicken MPR 46 protein was purified by affinity chromatography on phosphomannan gel (PM gel); excluding the NaCl in column buffer (page 35, section 2.2.5). 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.

3.2.8. Immunoblotting

Samples were analyzed by SDS-PAGE under reducing conditions as described (Laemmli, 1970). Gels were stained using the silver staining method (Blum et al., 1987). For immunological detection, the method described earlier was followed (page 36, section 2.2.6).

3.2.9. Enzyme assays

Equal amounts of the cell lysate from the mock transfected cells ($mpr^{(-/-)}$) and chicken MPR 46 expressing cells ($mpr^{(-/-/MPR\ 46)}$) were taken for enzyme assays. The activities of β-hexosaminidase, α-galactosidase, α-mannosidase, α-fucosidase were analyzed using *p*-nitrophenyl derivatives of the respective substrates and arylsulfatase A was assayed using 4-nitrocatechol sulfate dipotassium salt, as below. The sample taken was finally made up to 500 μl volume with sodium acetate buffer pH 5.0. To this 100 μl of corresponding substrate was added and incubated at 37^0C for 15 min. The reaction was stopped with 500 μl of 0.2 M Na_2CO_3 pH 9.2, the color developed was read at 405 nm. Activity was expressed in IU/ml = (A_{405}x1/ t x Vx ε) where t = time of incubation (15 min), ε = Extinction coefficient of p-nitrophenol (18.5), A_{405} = Absorbance at 405nm, V = Volume of sample in ml. The activities were then calculated for mg of protein. Each of the enzyme activity was assayed as quadruples and plotted using Sigma plot 9.0 taking the averages and standard deviation for the error bars.

3.2.10. Immunofluorescence

The chicken MPR 46 expressing cells ($mpr^{(-/-/MPR\ 46)}$) were analyzed for the presence and distribution of MPR 46 protein by immunofluorescence using MSC1

antibody. The cells were fixed in 4% paraformaldehyde in PBS buffer for 6 min at room temperature, washed, and permeabilized with 0.2% saponin in PBS for 6 min. The cells were later washed and blocked with 5% BSA in PBS for 30 min at room temperature before incubation with Lamp-1 antibody (1:100 dilution) followed by MSC1 antibody, 2 µg/ml in 5% BSA in PBS for 1 h. Subsequently the cells were washed 5-6 times with PBS, blocked with BSA as above, incubated with fluorescent tagged anti-rabbit IgG-Cy3 (Chem Bio) followed by anti-mouse IgG-FITC (Bangalore Genei) for 1 h at room temperature. Finally the cells were washed extensively and observed under confocal microscope. The $mpr^{(-/-)}$ MEF cells expressing chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) were also analyzed for its ability to bind and sort cathepsin D using cathepsin D antiserum by immunofluorescence. Lysosomes were labeled by Lamp-1 antibody. After fixation and permeabilization the cells were incubated with cathepsin D antiserum (1:250 dilution) and Lamp-1 antibody (1:100 dilution) in 5% BSA, followed by incubation with anti-rabbit IgG-Cy3 followed by anti-mouse IgG-FITC (Bangalore Genei).

3.2.11. Quantification of cathepsin D missorting

About 45 µg of total protein from the cell lysates and equal amounts of the medium from 12 h cultured chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells, mock transfected $mpr^{(-/-)}$ MEF cells was subjected to immunoblotting with cathepsin D antiserum. The blot was developed with ECL reagent and the signal intensities of the bands were quantified by densitometer (GS 800 DOC from Biorad).

3.3. RESULTS

3.3.1. Expression of chicken MPR 46 protein in $mpr^{(-/-)}$ MEF cells

The purity and integrity of the isolated total RNA was checked on a 1% agarose gel under denaturing conditions (Fig 3.4.1A). Using the primers mentioned under methods an 840 bp chicken MPR 46 gene was amplified (Fig 3.4.1B lane 2) which was then cloned into mammalian expression vector pcDNA.6/V5-His A. The presence of insert was confirmed by double digestion of the construct (Fig 3.4.1C lane 4). Sequence was confirmed by automated DNA sequencing.

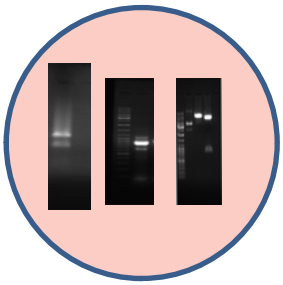
Fig 3.4.2 shows the comparison of MPR 46 protein sequences of different vertebrate receptors. The chicken sequence obtained is 81% similar to the human protein. It has 23 amino acid signal sequence, six cysteine residues in its extracytoplasmic region that are involved in disulfide bridge formation to form properly folded receptor, a single transmembrane domain and a highly conserved cytoplasmic tail. After transfection, stable transformants were selected in Blasticidin S containing medium. Of the 20 clones selected, expression level of MRP 46 protein in each clone was tested by RT-PCR taking equal amounts total RNA from different clones, CEF cells, MEF cells and mock transfected $mpr^{(-/-)}$ MEF cells (Fig 3.4.3). The clone expressing the physiological level of MPR 46 protein was expanded further (Fig 3.4.3B lane 6).

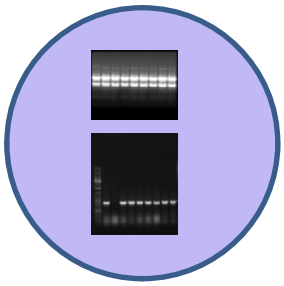
The lysate from the stable cells and mock transfected cells was subjected to SDS-PAGE and the protein detected by subsequent immunoblotting with MSC1 antibody and goat MPR 46 antiserum. The antibodies recognized two polypeptides considered to represent the monomer (M) and homodimer (D) of MPR 46 (Fig 3.4.4A lane 2 and Fig 3.4.4B lane 2, respectively). The mock transfected cells did not show any bands (Fig 3.4.4A lane 1 and Fig 3.4.4B lane 1, respectively). The expressed protein was purified by affinity chromatography on phosphomannan gel (PM). The protein bound to the PM gel was specifically eluted with 5 mM mannose 6-phosphate (Fig 3.4.4C lane 3) but not with 5 mM glucose 6-phosphate (Fig 3.4.4C lane 2). This establishes that chicken MPR 46 binds specifically to phosphomannan gel like the other known MPR 46 proteins in a mannose 6-phosphate dependent manner. Removal of metal ions from the column buffer resulted in failure to bind on the PM gel (Fig 3.4.5). Fig 3.4.6, shows the expression and distribution of chicken MPR 46 protein as examined by immunofluorescence using MSC1 (receptor antibody) and Lamp-1 antibody (Lamp-1 is a trans-membrane protein enriched in late endosomes and lysosomes) in comparison with the mock transfected cells. The expressed chicken MPR 46 protein and its localization mainly at the perinuclear region can be clearly seen (Fig 3.4.6 panel B).

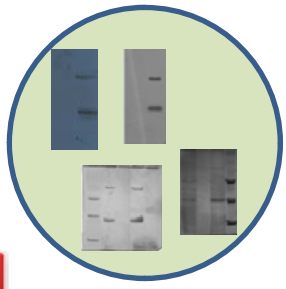
3.3.2. Sorting of lysosomal enzyme cathepsin D

The functional analysis of the expressed receptor protein can be assessed by monitoring the transport of lysosomal enzymes in the cells expressing chicken MPR 46. For this purpose, we first analyzed some of the lysosomal enzyme activities in the cells expressing the chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) protein and found that the cell lysates have retained significant enzyme activities (Fig 3.4.7). The enzyme activities have increased in the range of 2.5 to 4.5 times suggesting that the expressed receptor is able to mediate transport of the newly synthesized lysosomal enzymes. In mammals it has been well established that several of the lysosomal enzymes including those that were tested in this study are sorted to lysosomes by the mannose 6-phosphate receptors (Matzner et al., 1996). To conclusively establish this function of the receptor, we chose cathepsin D as this has been shown by us recently as the ligand for goat and zebra fish MPR 46 proteins. This enzyme was detected and localized in the chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells (Fig 3.4.8B) and mock transfected cells (Fig 3.4.8F) using cathepsin D antiserum. The lysosomes in the same cells were stained by Lamp-1 antibody, chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells (Fig 3.4.8A) and mock transfected cells (Fig 3.4.8E). Extensive colocalization of cathepsin D with Lamp-1 was observed in chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells (Fig 3.4.8C). Moreover the concentration of the cathepsin D was much higher in the chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells (Fig 3.4.8B) compared to the mock transfected cells (Fig 3.4.8F). This confirms the sorting ability of the expressed chicken MPR 46 protein. In order to quantify the level of sorting efficiency, a western blot was done and the signal intensities were calculated using densitometer (GS 800 DOC from Biorad). Equal amounts of protein from cell homogenates and 12 h culture medium of both chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells and mock transfected cells was analyzed for cathepsin D levels by immunoblotting. The blot was developed by ECL reagent and the densitometric analysis showed that 50.2% of cathepsin D (Fig 3.4.9A lane C) is found intracellularly in mock transfected cells and was increased to 92.3% in chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells (Fig 3.4.9B lane C). The percentages given are the average of three individual experiments. The three forms of cathepsin D precursor (P),

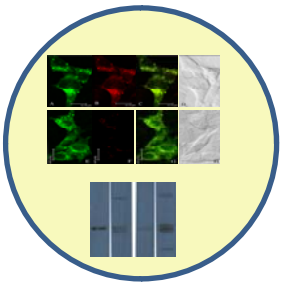
intermediate (I), and mature (m) were also observed similar to that obtained for human and zebrafish in our earlier results (Suresh et al., 2005).

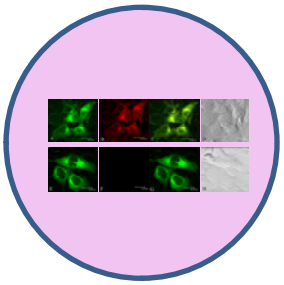






FIGURES 3.4





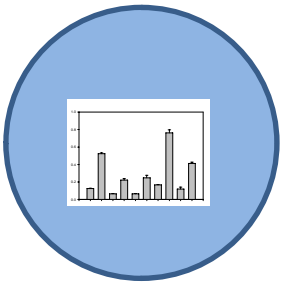


Figure 3.4.1: (**A) Agarose gel electrophoresis of the isolated chicken liver total RNA (5 µg)**. **(B) Amplification of chicken MPR 46 gene using RT product as the template**; lane 1, mixed DNA ladder (10,000 to 100 bp), lane 2, specific amplification of 840 bp. **(C) Restriction analysis of the clone**, lane 1, mixed DNA ladder (10,000 to 100 bp), lane 2, undigested chicken MPR 46 gene in pcDNA.6/V5-His A, lane 3, single digested chicken 46 in pcDNA.6/V5-His A (with EcoRI), and lane 4, double digested chicken 46 in pcDNA.6/V5-His A (with EcoRI and XhoI) the release of the 840 bp fragment is indicated by the arrow.

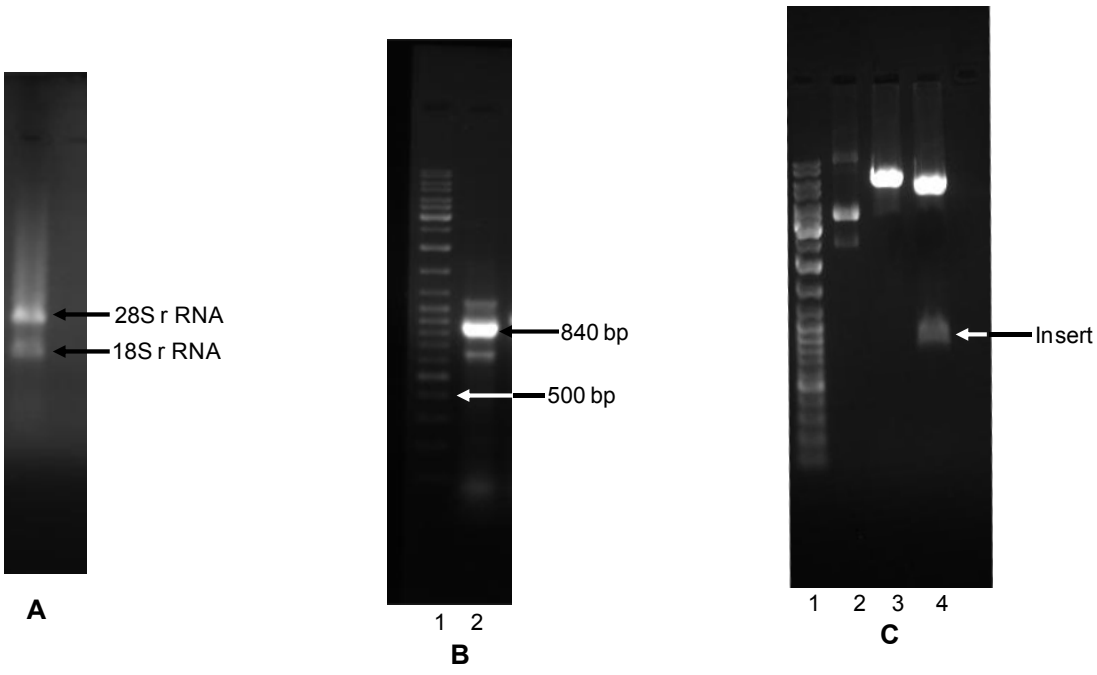


Figure 3.4.1

Figure 3.4.2: **Amino acid sequence alignment of MPR 46 from different species**:(▼) indicates the signal peptidase cleavage site; (●) indicates the conserved residues essential for M6P-binding; (★) indicates residues where mutation resulted in reduced binding; N in bold italics indicates a potential N-glycosylation site; numbers 1, 2, 3 indicate the cysteines paired in disulfide bonds; characters shown in bold indicate the conserved acidic dileucine motif DxxLL interacting with sorting GGAs; the underlined sequence represents the trans-membrane domain; the bold italicised characters mark the tyrosine-dependent endocytosis motif YRGV; (*) marks identical amino acid residues; (:) related amino acid residues; (·) predominantly the same amino acid residue. (BOV, bovine; GOT, Goat; HUM, Human; MUS, Mouse; CHI, Chicken; XEN, *Xenopus*; FUG, Fugu fish; XIP, *Xiphophorus*; KIL, killifish, partial sequence; ZEB, zebrafish).

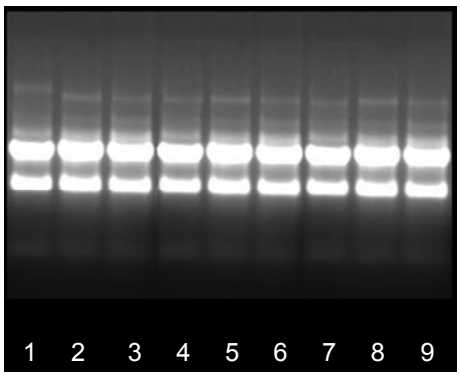
Figure 3.4.2

Sequence Alignment

```
CLUSTAL W (1.8) multiple sequence alignment

                      1                              1                       54
BOV46           --MMSPLHSSWRTGLLLLLLFSVAVRESWQ▼TE-EKTCDLVGEKGKESEKELALLKRLTPL
GOT46           ---MSPLHSSWRTGLLLLLLFSMAVRESWQ▼TE-EKTCDLVGEKGKESEKELALLKRLTPL
HUM46           ---MFPFYSCWRTG-LLLLLLAVAVRESWQ▼TE-EKTCDLVGEKGKESEKELALVKRLKPL
MUS46           ---MFPFSGCWRTELLLLLLLAVAVRESWQ▼IE-EKSCDLVGEKDKESKNEVALLERLRPL
CHI46           ------MSSHCHTSAVLVVFMALAAGVGA▼EPLSEKSCDVVGDESTESQMEKALLKKLEPL
XEN46           -----      MCAPLWCVSIAALLAIAAG▼ENLVDSCQLVGG-DRKSVTEQQLLAKLAPL
FUG46           --MVNSYTRGSLLMWALALWVILDGGPVCA▼TNSTQSCKLLSE------SDRKVLSQLEPL
XIP46           --MVNSYTRGSLLMWALALWVILDGGPVCA▼TNSTQSCKLLSE------SDRKVLSQLEPL
KIL46           MKLFRIARRGYVLLWALLAQLVLC▼RSGVFAGDGTKACVLAHE----TVSERKVLDRLEPL
ZEB46           ----------MLLSVRIITPLFVLFAGVQA▼RFNTSNCKLVSD----SESQRKALRLLEPL
                                :   . :              * :          :   :  * **

              55                ★      1               ●                    110
BOV46           FNKSFESTVGQSP-DMYSYVFRVCREAGNHSSG--AGLVQINKSNGK---ETVVGRFNET
GOT46           FNKSFESTVGQSP-DMYSYVFRVCREAGNHSSG--AGLVQINKSNGK---ETVVGRFNET
HUM46           FNKSFESTVGQGS-DTYIYIFRVCREAGNHTSG--AGLVQINKSNGK---ETVVGRLNET
MUS46           FNKSFESTVGQGS-DTYSYIFRVCREASNHSSG--AGLVQINKSNDK---ETVVGRINET
CHI46           SQIRFNTTVEIGTTENYAYHFRVCREVNSSLHDF-AGLVQMDRQSGK---TTVIGRINET
XEN46           KGKRFEAKTQEGS-DIYTYTFVVCGRVNNDSKSTNEGLVQSKDGSKD---TSVIGRINDT
FUG46           AHQNFSGQSNNE---NYTYVFQLCGDAGG-TSG--AGVIQIDNKKKE-AKPTVIGTYNLT
XIP46           AHQNFSGQSNNE---NYTYVFQLCGDAGG-TSG--AGVIQIDNKKKE-AKPTVIGTYNLT
KIL46           AFKNLTVESKNEK-DSYTYVFQLCGDAGG-VPG--AGVIQKNMKTPEKPIVTNIGSYKAT
ZEB46           TNQNFTTEGQEK--EKYSYIFQVCGDAGG-VKN--AGLIQQEKGGKT----IRIGDYSKT
                    :           * * * :*   ...   .   *::* .           :*  . *

             111                     ★2    ●       3             ● ★     2 ●170
BOV46           QIFNGSNWIMLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFY
GOT46           QIFNGSNW-MLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFY
HUM46           HIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFY
MUS46           HIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLAANFNPVSEERGKVQDCFY
CHI46           QVFNGSDWIMLIYKGGDSYGRHCSGEKRRAVIMISCKRGITASSFSIISEEREKEQDCFY
XEN46           HIINGSDWIMLYYRSGDKYDTHCNNEARKAMVMISCNKGTVGDGFTVIQEERNKSSECFY
FUG46           QAIGESNWVVLTYTYNNKHGS-CSKDASQAQIMIFCNREVDESPLHVIQENSERENHCLY
XIP46           QAIGESNWVFLTYTYNNKHGS-CSKDASRAQIMIFCNREVDESPLHVIQENSEREDHCLY
KIL46           KAIGGSDWVMLIYEQGDKYDSHCQKEARKAIIMISCSKNTDVGALEVILEENERDTDCFY
ZEB46           VATAGSDWVLLIYEGGEKYDSHCSSEERKAMIMISCSSSSK-SAFSVVMEENQKQKNCYY
                     *:* .* *  .:.:.  *  :  :* :** *.       :  : *:   :   .**

             171         3                                                  230
BOV46           LFEMDSSLACSPEISHLSVGSILLVTLASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFW
GOT46           LFEMDSSLACSPEISHLSVGSILLVTFASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFW
HUM46           LFEMDSSLACSPEISHLSVGSILLVTFASLVAVYVVGGFLYQRLVVGAKGMEQFPHLAFW
MUS46           LFEMDSSLACSPEVSHLSVGSILLVIFASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFW
CHI46           LFEMDSSVACPAEDSHLSTGSILLITFSALVTVYIVGGFLYQRLIVGAKGMEQFPHFAFW
XEN46           LFEMDSSLACPPEESHLSAGSILLIVFAVLVAVYLIGGFLYQRFVVGAKGMEQFPNITLW
FUG46           LFRLDSSAVCPALQSKLSAGSIILIIGFCLLTVYLVGGFLYQRMIVGAKGMEQFPNYAFC
XIP46           LFRLDSSAVCPALQSKLSAGSIILIIGFCLLTVYLVGGFLYQRMIVGAKGMEQFPNYAFW
KIL46           LFELDSSAVCPPVESRLSAGSIILIIIFCLVAVYLIGGFLYQRLIV--------------
ZEB46           LFELDTTAVCPAVSSKLSAGSIVLIVVISSLTVYIIGGFLYQRLVVGAKGVEQFPNFAFW
                **.:*:: .*..  *:**.***:*:     ::**::*******::*

             231                                                 284
BOV46           QDLGNLVADGCDFVCRSK-PRNVPAAYRGVGDDQLGEESEERDDHLLPM
GOT46           QDLGNLVADGCDFVCRSK-PRNVPAAYRGVGDDQLGEESEERDDHLLPM
HUM46           QDLGNLVADGCDFVCRSK-PRNVPAAYRGVGDDQLGEESEERDDHLLPM
MUS46           QDLGNLVADGCDFVCRSK-PRNVPAAYRGVGDDQLGEESEERDDHLLPM
CHI46           QDLGNLVADGCDFVCRSK-PRNVPAAYRGVGDDQLGDESEERDDHLLPM
XEN46           QELGNLSADGCDFVCRSR-PRTSETAYRGVGEDQLGEEPEERDDHLLPM
FUG46           VEVGNLAADGCDFVCRSR-TREEVPTYRGVSSEPSEDDPEERDDHLLPM
XIP46           VEVGNLAADGCDFVCRSR-TREEVPTYRGVSSEPSEDDPEERDDHLLPM
KIL46           -------------------------------------------------
ZEB46           SEIGNLSADGCDFVCRSRGNREEPPTYRGVGTEPLGEEPEERDDHLLPM
```

Figure 3.4.3: About 5 µg of total RNA from each of the clone and the CEF, MEF and $mpr^{(-/-)}$ MEF cells was taken and RT reaction was done. From the RT product 2 µl was used for the PCR reaction in the next step and run on gel. **(A) Ethidium bromide stained RNA gel**; lane 1, CEF cells total RNA, lane 2, MEF cells total RNA, lane 3, mock transfected $mpr^{(-/-)}$ MEF cells total RNA, lanes 4 to 9 are chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) expressing clones 1 to 6, total RNA. **(B) The RT-PCR product run on 1% agarose gel**; lane 1, Mixed DNA ladder, lane 2, clone 1, lane 3, mock transfected $mpr^{(-/-)}$ MEF cells, lane 4, MEF cells, lane 5, CEF cells, lane 6 to 10 are ($mpr^{(-/-/MPR\ 46)}$) clones 2 to 6.

A



B

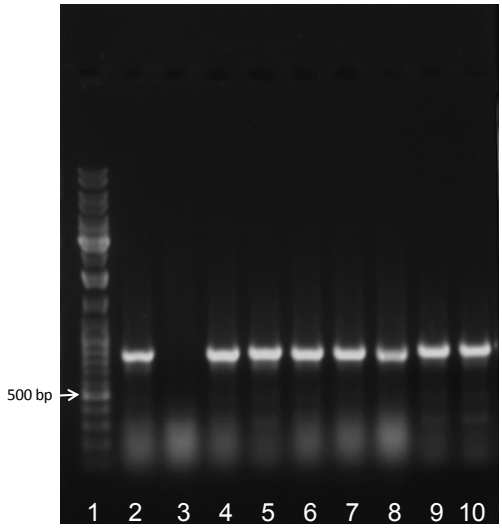


Figure 3.4.3

Figure 3.4.4: **(A and B); Western blot analysis of the expressed protein**, Lane 1, mock transfected cell lysate, lane 2, lysate from the cells expressing chicken MPR 46 protein, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with MSC1 antibody (2 µg/ml) (A), goat MPR 46 antiserum (B). Blots were developed with ECL reagent; (M) and (D) represent the monomeric and homodimeric form of the receptor. **(C) Purification of expressed chicken MPR 46 protein on PM gel.** Proteins were separated on 10% SDS-PAGE and silver stained; lane 1, wash, lane 2, glucose 6-phosphate eluate, lane 3, mannose 6-phosphate eluate. (M) and (D) represent the monomeric and homodimeric forms, lane 4, molecular weight markers.

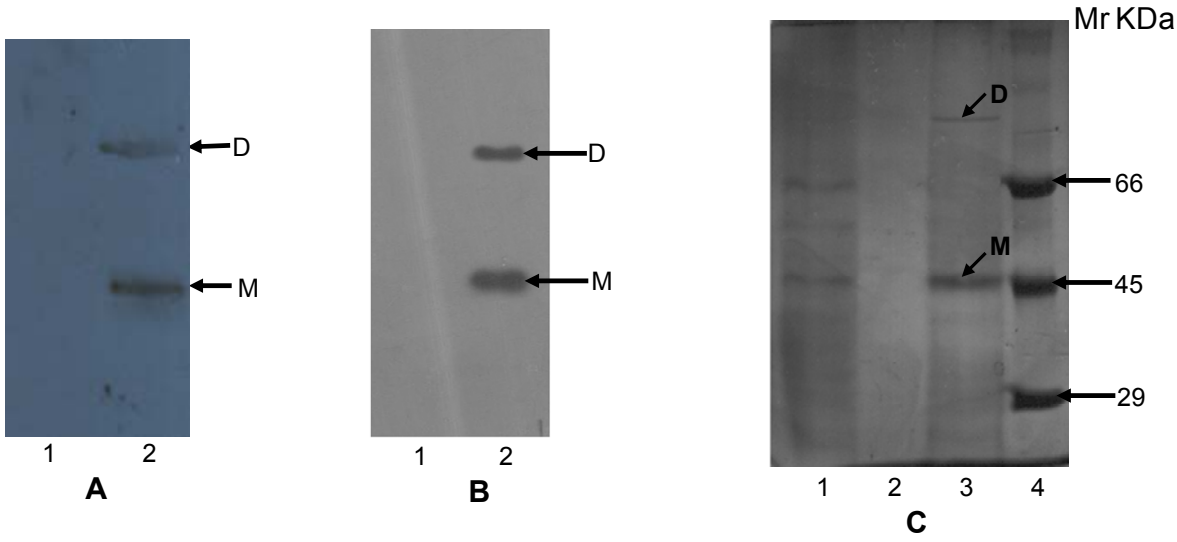


Figure 3.4.4

Figure 3.4.5: **Requirement for divalent cations by chicken MPR 46 protein to bind PM gel.** An aliquot of the purified, acetone precipitated sample was loaded on a 1 ml PM gel column, one equilibrated with the buffer containing divalent cations and the other without cations. The gel after washing, eluted with 5 mM M6P in respective column buffers. Both the unbound and eluted fractions were loaded on SDS-PAGE. Lane 1, molecular weight marker, lanes 2 and 3 are eluate and unbound in presence of divalent cations. Lanes 4 and 5 are unbound and eluate in the absence of divalent cations.

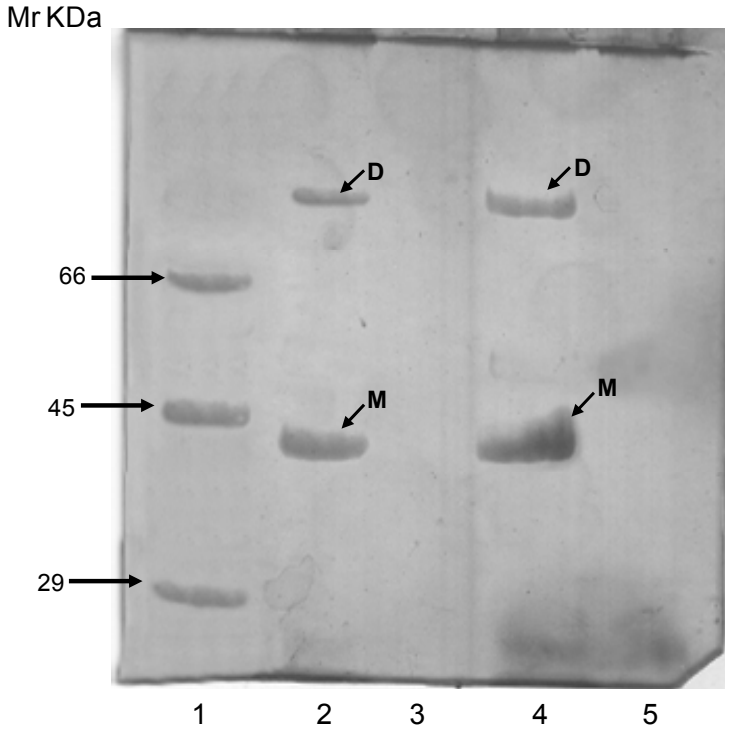


Figure 3.4.5

Figure 3.4.6: **Localization of MPR 46 protein**; (A) and (E) incubation with Lamp-1 followed by FITC (green) conjugated secondary antibody, of $mpr^{(-/-)}$ MEF cells expressing chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) and mock transfected $mpr^{(-/-)}$ MEF cells, respectively. (B) and (F) Incubation with MSC1 antibody followed by Cy3 (red) conjugated secondary antibody, of chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells and mock transfected $mpr^{(-/-)}$ MEF cells, respectively. (C) Merged image of A and B, (G) merged image of (E) and (F). (D) and (H) are the corresponding transmission images. Bar in the top panel is 14.43 μm and the bottom panel is 15.75 μm.

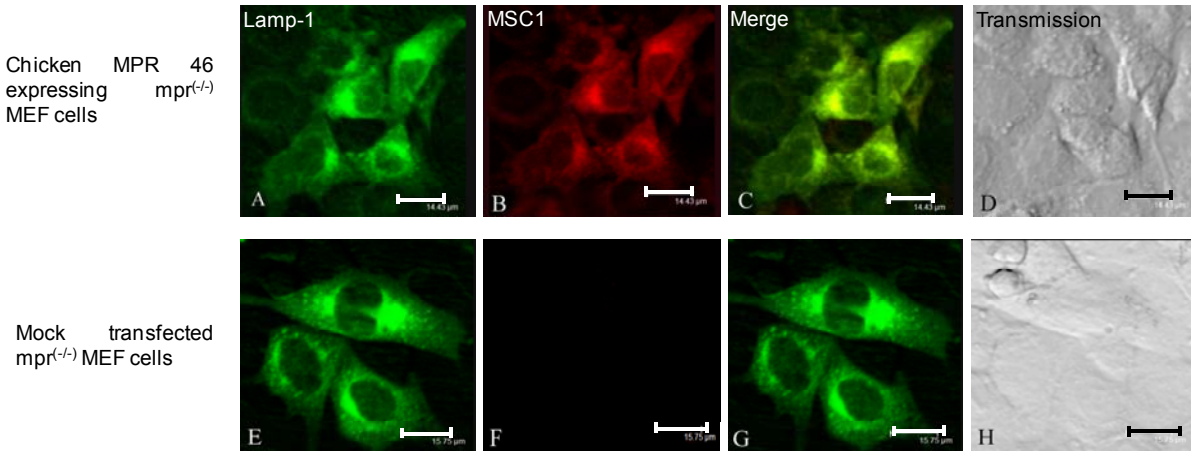

Chicken MPR 46 expressing mpr(-/-) MEF cells
Lamp-1
MSC1
Merge
Transmission
A
B
C
D
Mock transfected mpr(-/-) MEF cells
E
F
G
H

Figure 3.4.6

Figure 3.4.7: **Lysosomal enzyme activities.** The enzyme activities from the cell lysates of mock transfected (Mock) and chicken MPR 46 (C46) expressing ($mpr^{(-/-/MPR\ 46)}$) cells were tested with the respective chromogenic substrates. H, G, M, A and F represent β-hexosaminidase, α-galactosidase, α-mannosidase, arylsulfatase A and α-fucosidase enzyme activities, respectively.

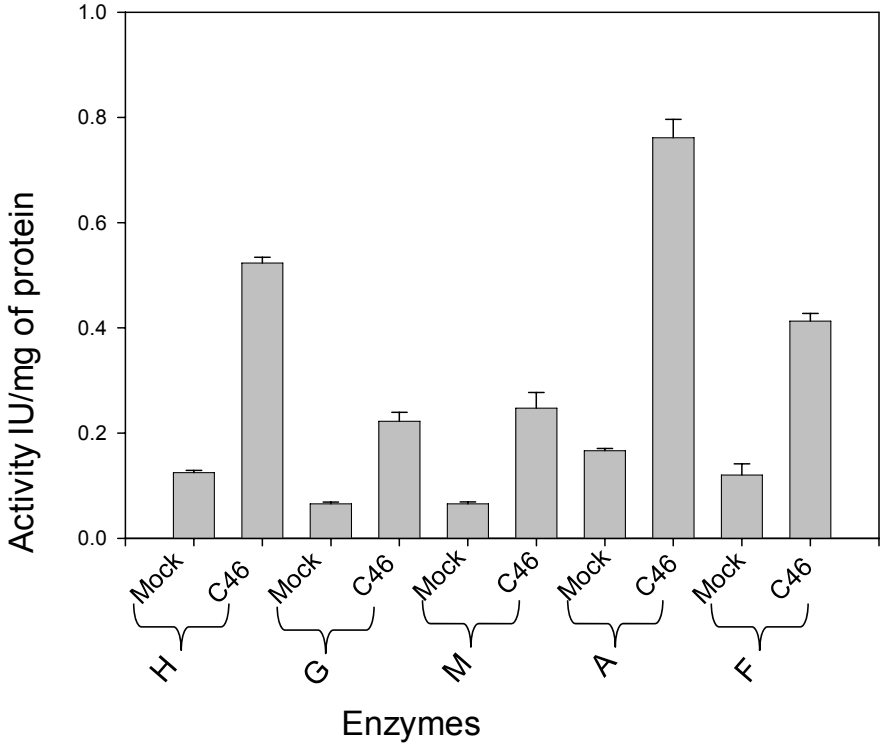

1.0
0.8
0.6
0.4
0.2
0.0
Activity IU/mg of protein
Mock
C46
Mock
C46
Mock
C46
Mock
C46
Mock
C46
H
G
M
A
F
Enzymes

Figure 3.4.7

Figure 3.4.8: **Intracellular retention and localization of cathepsin D**; (A) and (E) incubation with Lamp-1 followed by FITC (green) conjugated secondary antibody, of $mpr^{(-/-)}$ MEF cells expressing chicken MPR 46 ($mpr^{(-/-/MPR\ 46)}$) and mock transfected $mpr^{(-/-)}$ MEF cells respectively. (B) and (F) incubation with cathepsin D antiserum followed by Cy3 (red) conjugated secondary antibody, of chicken MPR 46 expressing ($mpr^{(-/-/MPR\ 46)}$) cells and mock transfected $mpr^{(-/-)}$ MEF cells, respectively. (C) Merged image of (A) and (B), clearly shows the extensive co-localization of Lamp-1 and cathepsin D, thus supporting the function of chicken MPR 46 protein to bind and target cathepsin D to lysosomes. (G) Merged image of (E) and (F). (D) and (H) are the corresponding transmission images. Bar in the top panel is 12.74 μm and the bottom panel is 16.41 μm.

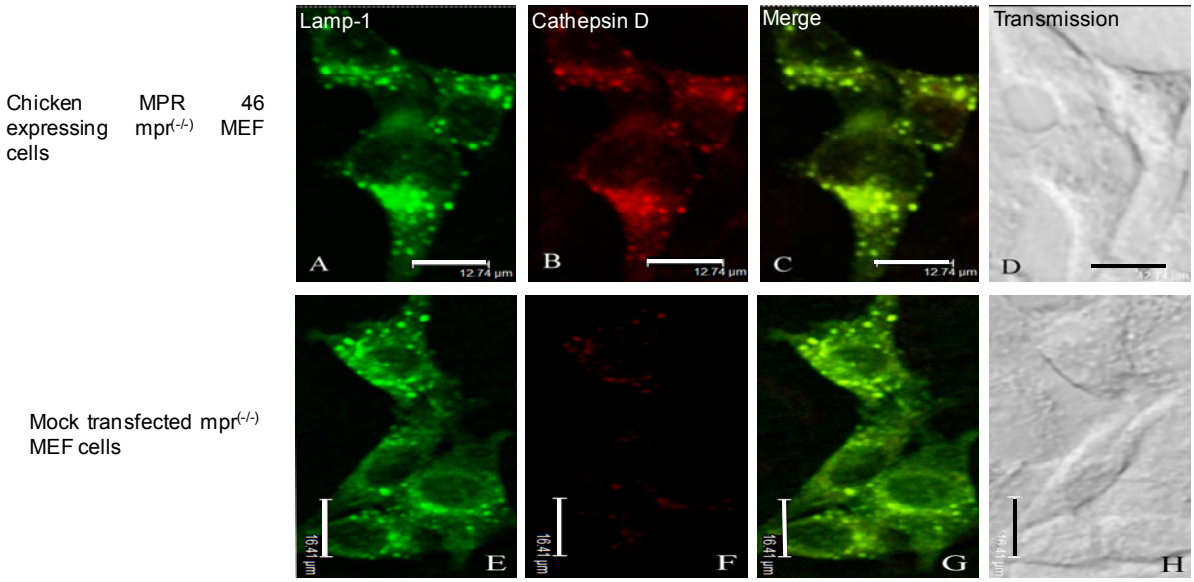

Chicken MPR 46 expressing mpr(-/-) MEF cells
Mock transfected mpr(-/-) MEF cells
Lamp-1
Cathepsin D
Merge
Transmission
A
B
C
D
E
F
G
H
12.74 µm
16.41µm

Figure 3.4.8

Figure 3.4.9: **Cathepsin D blot.** (A) Quantification of cathepsin D protein present in the cell lysate (C) and the 12 h culture medium (M) from the mock transfected $mpr^{(-/-)}$ MEF cells. (B) Quantification of cathepsin D present in chicken MPR 46 protein expressing ($mpr^{(-/-/MPR\ 46)}$) cell lysate (C) and the 12 h culture medium (M). The three forms of cathepsin D precursor (P), intermediate (I), and mature (m) are shown by arrows.

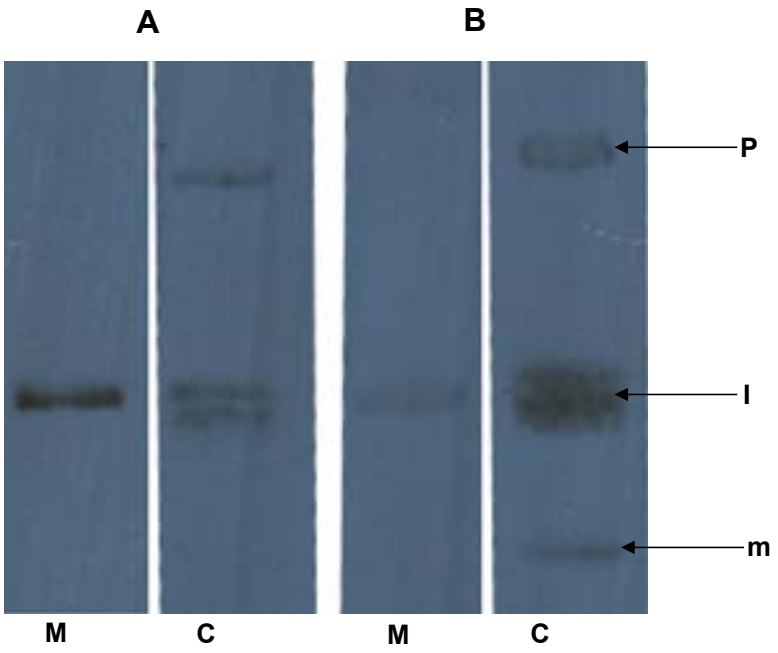


Figure 3.4.9

3.5. DISCUSSION

It is well established from studies carried out by others using mammalian species, and more recently by us using the goat (mammalian) and the early vertebrate (zebrafish), that the key function of mannose 6-phosphate receptor MPR 46 in these species is to mediate sorting of newly synthesized lysosomal acid hydrolases and the receptor recycles between TGN and late endosomes. In a previous study it was found that when the chicken MPR 46 protein sequence derived from the two partially overlapping sequences found in the database was aligned with other vertebrate receptors, it exhibited extensive homology (Suresh et al., 2005). In the present study for the first time the full length chicken MPR 46 gene was cloned from the total RNA of the liver tissue, and its sequence was found to be identical to the one found in the database and shown by us earlier (Suresh et al., 2005). The residues that have been shown to be essential for mannose 6-phosphate binding in other species Tyr^{71}, Gln^{93}, His^{132}, Arg^{138}, Glu^{160}, and Tyr^{170} are also conserved in chicken. The cytoplasmic tails of the chicken and human MPR 46 differ by only three conservative amino acid substitutions. The sequence ^{221}MEQFPHFAFW^{230} required for binding to AP2 (Adaptor protein2), ^{239}DGCDFVCRSKPRNVP^{253} required for binding to AP1 (Adaptor protein1) is also present in chicken MPR 46 protein (Ghosh et al., 2003). The sequence ^{256}YRGVGDDQLGDESEERDDHLL^{282} comprising the tyrosine-dependent endocytosis motif YRGV (position 256-259) and the acidic cluster dileucine motif DxxLL (position 278-282), that are known to be involved in the recognition of the sorting GGAs (golgi associated gamma adaptin ear containing ARF binding proteins) AP1, AP2 and phosphofurin acidic cluster-sorting protein-1 (PACS-1) which recognizes phosphoserine residue and also interacts with AP1 (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001; Ghosh et al., 2003; Bonifacino and Raul Rojas, 2006) are also conserved. All these properties and the specific motifs exhibited by the chicken receptor and other known vertebrate receptors, highlight the fact that the MPR 46 is conserved throughout the vertebrates.

Although both receptors were identified from chicken and that the chicken MPR 300 was also shown to transport lysosomal enzymes (Zhou et al., 1995; Matzner et al., 1996), the function of MPR 46 has not been elucidated, and little is known about its role

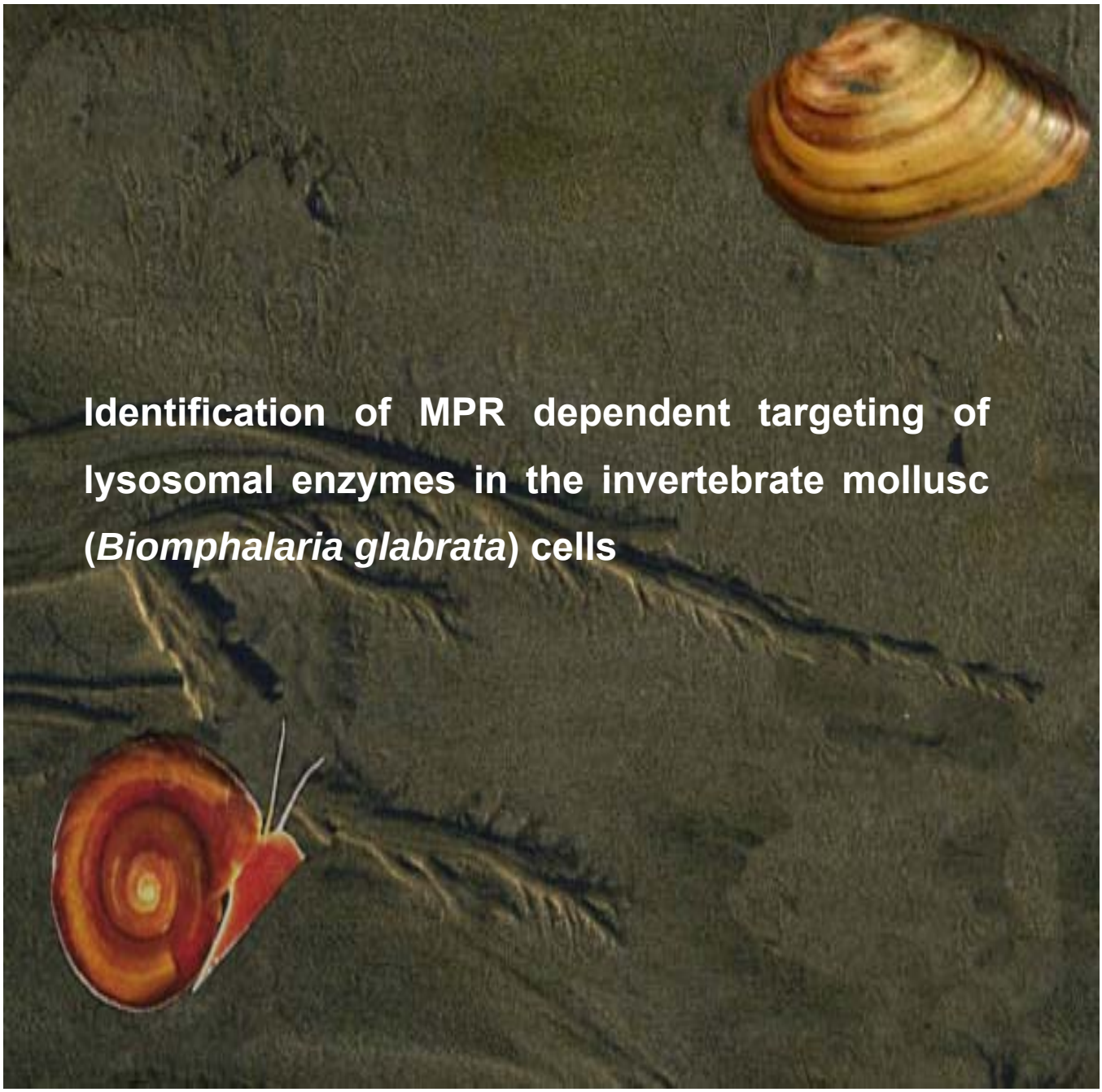
in lysosomal sorting process. Therefore a detailed study was undertaken here to establish the function of the MPR 46 protein which can pave way to understand the biogenesis of lysosomes in the highly evolved non-mammalian vertebrate, chicken. Since we have already achieved success in expressing the goat MPR 46 cDNA in $mpr^{(-/-)}$ MEF cells, the same strategy was used to express the chicken MPR 46 cDNA and the function of the protein extensively characterized. The expressed chicken MPR 46 protein partially corrects the missorting of lysosomal enzymes in $mpr^{(-/-)}$ MEF cells that are devoid of both MPR 300 and MPR 46 receptor proteins. These cells secrete 80% of the newly synthesized lysosomal enzymes which can be corrected by expressing either or both the MPRs, thus becoming valuable in analyzing the functional role of the MPRs (Köster et al., 1993).

The two MPR proteins identified so far (cation dependent MPR 46 and the cation independent MPR 300) are clearly distinguished by their ability to bind phosphomannan gel in the presence and absence of divalent metal ions, respectively. The chicken receptor expressed also binds PM gel only in the presence of divalent metal ions. It is interesting to note that the chicken receptor identified from the chicken embryonic fibroblasts (Matzner et al., 1996; Siva Kumar et al., 1999) also exhibits the oligomeric nature and binds PM gel only in presence of the divalent metal ions.

In summary this is the first report demonstrating the function of the chicken MPR 46 protein. Several lines of evidence support our studies, (i) the cloned full length chicken liver MPR 46 cDNA can be stably expressed in $mpr^{(-/-)}$ MEF cells (like the goat and the zebrafish proteins), (ii) the expressed protein can be affinity purified on a PM gel in the presence of divalent metal ions and exhibits typical biochemical and immunological characteristics similar to the other vertebrate receptors, (iii) furthermore, immunofluorescence studies clearly establish that the expressed MPR 46 corrected the missorting of cathespin D in $mpr^{(-/-)}$ MEF cells, as was recently found for zebrafish and goat protein (Suresh et al., 2005; Praveen Kumar et al., 2007). Our results on the chicken MPR 46 thus provide new insights, and make the study complete on the chicken MPR 46 that reveals structural and functional homologies among the different vertebrate MPR 46 proteins suggesting their evolutionary significance. With the results presented in the study it can be concluded that in each of the representative vertebrate

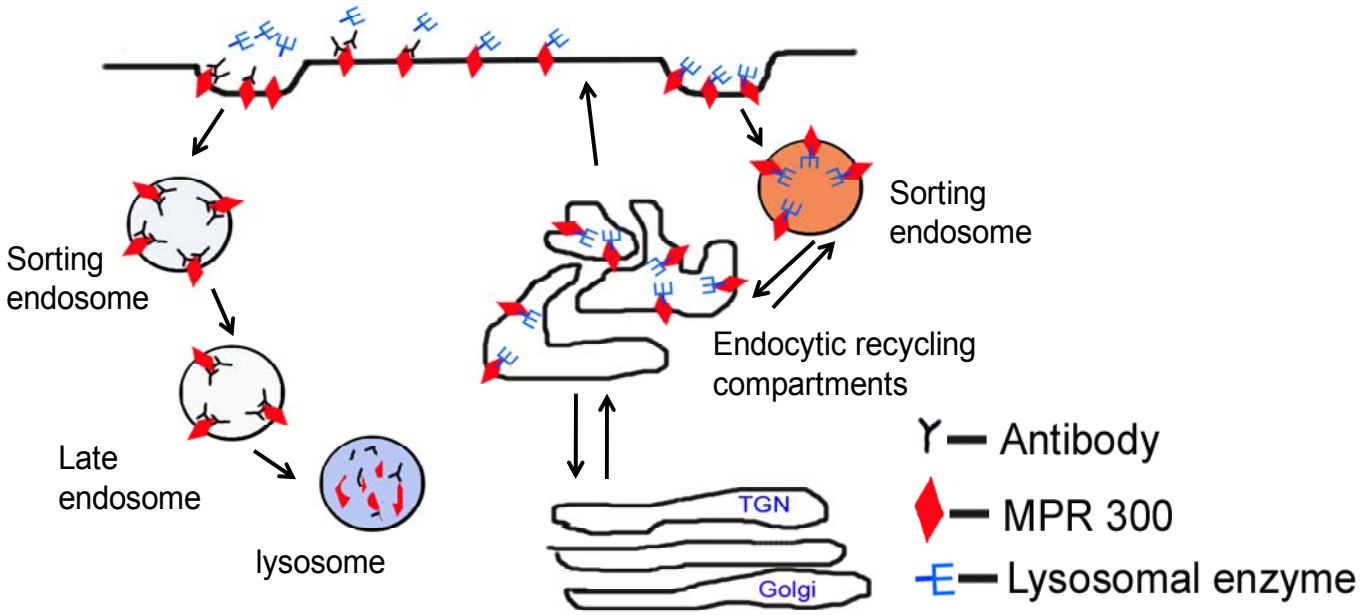
species (fish to mammals) the MPR 46 protein exhibits structural similarities and functions in lysosomal enzyme sorting.

CHAPTER 4

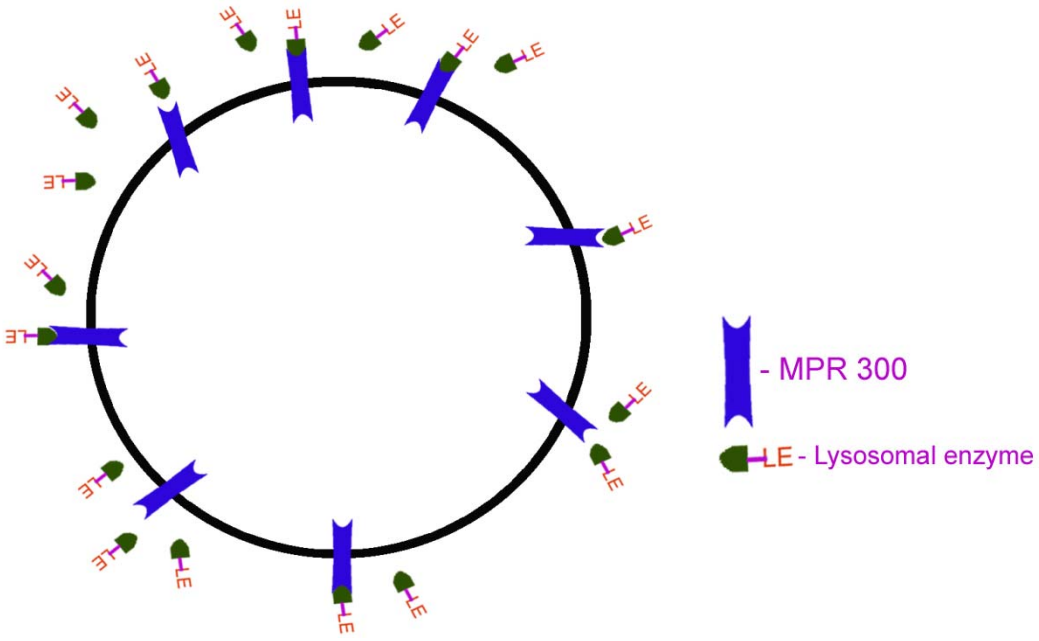


Identification of MPR dependent targeting of lysosomal enzymes in the invertebrate mollusc (*Biomphalaria glabrata*) cells

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



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



4A.1. INTRODUCTION

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

simultaneously the resultant effect on the fate of newly synthesized lysosomal enzymes was also analyzed. The M6P receptor proteins synthesized in cultured chinese hamster ovary cells and human fibroblasts persist with half-lives 6-32 h (Creek and Sly, 1983; Sahagian and Neufeld, 1983). In the present study, the observed half life of MPR 300 receptor protein (CIMPR) from *Bg* cell was ~ 13 h.

4A.2. MATERIALS AND METHODS

4A.2.1. Materials

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

4A.2.2. Lysosomal enzymes assays

The *Biomphalaria glabrata* (*Bg*) cells were cultured in the complete medium (22 ml of Schneiders Drosophila medium, 450 mg lactalbumin hydrolysate, 130 mg galactose, penicillin/Streptomycin (5 µg/ml), 1-2 mg phenol red, water 68 ml, pH 7.2 and 10% heat inactivated fetal bovine serum) in standard culture flasks at 28^{o}C without CO_2. Confluent cultures are detached from the surface by gushing with PBS (10 mM sodium

phosphate buffer pH 7.4, containing 150 mM NaCl); cell pellet was collected by centrifugation at 2991×g for 10 min. The pellet was suspended in 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge using a fixed angle 80Ti rotor at 161,280×g for 35 min. The supernatant obtained at this step is referred as acetate supernatant and was used for enzyme assays. The lysosomal enzyme activities of β-hexosaminidase, α-galactosidase, α-mannosidase, α-fucosidase were assayed using p-nitrophenyl derivatives of the respective substrates and arylsulfatase A was assayed using 4-Nitrocatechol sulfate dipotassium salt as described (page 54, section 3.2.9). Each of the enzyme activity was done in duplicates in two individual experiments and the results obtained were plotted using Sigma plot 9.0 taking the averages and standard deviation for the error bars.

4A.2.3. Metabolic labeling and immunoprecipitation

Biomphalaria glabrata (*Bg*) cells were cultured in 6 cm petri plates and labeled with 250 µCi of [^{35}S] methionine for 16 h as described (Siva Kumar and von Figura, 2002). The labeled cells were detached from the surface by gushing with TBS (20 mM Tris-HCl pH 7.4, 150 mM NaCl). The pellet was suspended in 0.5 ml of TBS containing 1 mM PMSF, 1 µg/ml leupeptin and pepstatin A. The cells were then solubilized by adding 0.5 ml of 2% Triton x-100, 0.1% NP-40 in TBS (X2 IP buffer) incubated on ice for 20 min and centrifuged at 14006xg for 15 min. To 300 µl of the supernatant obtained, 1% of respective antiserum, 20 µl of protein A-agarose slurry were added, volume finally made up to 500 µl with 1% Triton x-100, 0.05% NP-40 in TBS (IP buffer), incubated over night at 4^{o}C with rotation, centrifuged at 3502xg, and the supernatants were discarded carefully leaving few µl to avoid pipetting protein A-agarose beads. Resuspended in 500 µl of IP buffer and centrifuged again, the wash steps were repeated 3-4 times and the final supernatant was pipetted out completely with a narrow tip. Finally to the moist pellet of beads, 30 µl 2X SDS-PAGE sample buffer (reducing) was added, boiled at 95^{o}C for 5 min and loaded on to 10% SDS-PAGE. The gel was then fixed for 30 min in 50% methanol, 10% acetic acid and sequentially passed

through 1, 2 and 3 DMSO (Dimethylsulphoxide) bath for dehydration with 20 min incubation in each. The gel was finally incubated over night in 20% (w/v) PPO (2, 5 diphenyl oxazole) in DMSO; following day PPO on the gel was precipitated with a water wash (the gel turns white). The gel was placed in saran wrap dried and exposed to a Konica X-ray film or phosphorimager.

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

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

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

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

4A.2.6. Internalization of labeled exogenous lysosomal enzymes

Confluent cultures in 75-cm^2 flasks were incubated for 24 h in the labeling medium supplemented with protease inhibitors (aprotinin and leupeptin each 5 µg/ml concentration), 250 µCi of [^{35}S] methionine, 10 mM NH_4Cl (to induce the secretion of lysosomal enzyme precursors) (Hasilik and Neufeld, 1980). The secreted enzymes

were precipitated by adding 2.5 g of $(NH_4)_2$ SO_4, after stirring for 2 h at 4°C it was centrifuged in 8 x 50 ml fixed angle rotor at 17226xg, 20 min. The pellet obtained was dissolved in 1 ml of sterile water and dialyzed extensively against *Bg* cell medium without serum. To this dialyzed sample 0.25 ml of fetal bovine serum was added and the volume was adjusted to 5 ml with *Bg* medium, sterile filtered through 0.22 µm disposable syringe filter. These labeled secretions were offered to a confluent 75-cm^2 culture flask which was incubated previously with MS medium (350 mg NaCl, 16 mg KCl, 9 mg Na_2HPO_4 $2H_2O$, 45 mg $MgSO_4$ $7H_2O$, 53 mg $CaCl_2$ $2H_2O$, 5 mg $NaHCO_3$, 150 mg D-glucose, D-150 mg trehalose, dissolved in 100 ml of autoclaved water and sterile filtered) containing protease inhibitors for 24 h. At the end of additional 24 h incubation in the presence of labeled secretions, cells were detached and the cell lysates were subjected to immunoprecipitation for detecting internalized lysosomal enzymes as described above, followed by SDS-PAGE under reducing conditions, the protein bands were identified by fluorography and exposure to phosphorimager.

4A.2.7. Immuofluorescence studies

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

4A.3. RESULTS

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

To detect the lysosomal enzyme activities in *Bg* cells, acetate supernatant from the cells was assayed using chromogenic substrates. Of the five lysosomal enzymes assayed viz., β-hexosaminidase, α-galactosidase, α-mannosidase, α-fucosidase and arylsulfatase A, arylsulfatase A showed very high enzyme activity for the same amount of protein taken (Fig 4A.4.1). In order to identify the respective lysosomal enzymes, *Biomphalaria glabrata* (*Bg*) cells were metabolically labeled with 250 µCi of [^{35}S] methionine for 16 h and subjected to immunoprecipitation using the respective enzyme antiserum. The gels were treated for fluorography and developed by autoradiography.

Arylsulfatase A showed both precursor and mature forms (Fig 4A.4.2A), β-hexosaminidase showed two bands, a precursor form and a mature form below it (Fig 4A.4.2B), α-fucosidase showed a single band (Fig 4A.4.2C). The receptor proteins were also identified by using the *unio* MPR 300 antiserum and MSC1 antibody for detecting MPR 300 (Fig 4A.4.2D) and MPR 46 (Fig 4A.4.2E) proteins, respectively. An additional band seen above the MPR 46 protein in Fig 4A.4.2E could be a cross-reactive material or an aggregate form of the receptor.

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

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

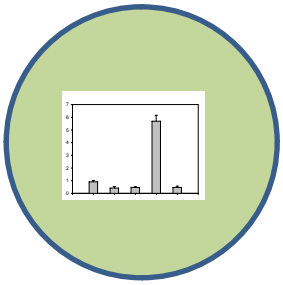
The effect of antiserum on missorting of endogenous lysosomal enzymes was analyzed in the cells that were metabolically labeled with [^{35}S] methionine for 24 h in the presence of preimmune serum and *unio* MPR 300 antiserum. After 24 h, both the cell

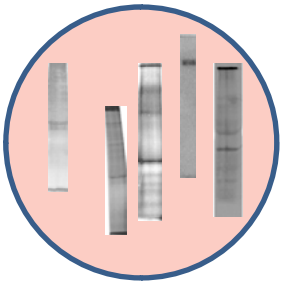
lysate and the medium from preimmune treated, *unio* MPR 300 antiserum treated were analyzed for the presence of arylsulfatase A (Fig 4A.4.4A), β-hexosaminidase (Fig 4A.4.4B) and α-fucosidase (Fig 4A.4.4C). From the results obtained it is evident that 80-85% of the tested enzymes were secreted into the medium treated with *unio* MPR 300 antiserum compared to the preimmune serum treated sample. The preimmune serum treated cells retained significant amounts of the enzymes but in the *unio* MPR 300 antiserum treated cells due to the depletion of the MPR 300 protein; the enzymes were secreted into the culture medium.

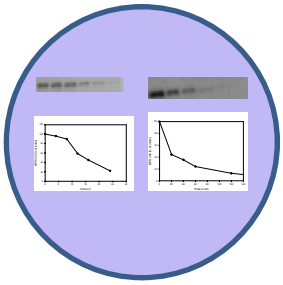
4A.3.3. Internalization of lysosomal enzymes and confocal microscopy

75-cm^2 confluent culture flasks were incubated for 24 h in the labeling medium containing 10 mM NH_4Cl which will induce the secretion of lysosomal enzyme precursors. These labeled secretions enriched in lysosomal enzyme precursors were concentrated by ammonium sulphate precipitation, dialyzed against *Bg* medium and offered to another 75-cm^2 culture flasks. Internalized arylsulfatase A (Fig 4A.4.5A), β-hexosaminidase (Fig 4A.4.5B) and α-fucosidase (Fig 4A.4.5C) from the cells that were incubated for 24 h with the labeled secretions was analyzed by immunoprecipitation. The detection of the respective enzymes reflects the rapid endocytosis of lysosomal enzymes by MPR 300 receptor protein present at the cell surface. The presence of mature forms in a large number of cases tested indicates that these have been processed in the destined lysosomes inside the internalized cells.

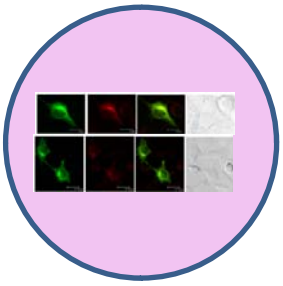
The *Bg* cells were also studied for the presence and distribution of MPR 300 (Fig 4A.4.6 top panel) and MPR 46 (Fig 4A.4.6 bottom panel) receptor proteins by immunofluorescence. Lamp-1 (Lysosomal associated membrane protein1, a trans-membrane protein enriched in late endosomes and lysosomes) was used as a marker protein. Endosomal distribution and partial colocalization of the receptor proteins with Lamp-1 can be seen.

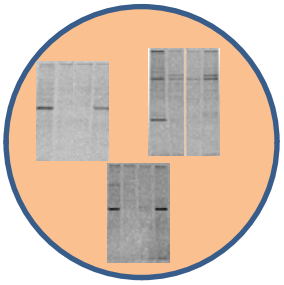






FIGURES 4A.4





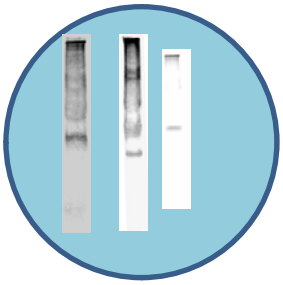


Figure 4A.4.1: **The Enzyme activities from the *Bg* cell acetate supernatant with the respective chromogenic substrates**. H, G, M, A and F represent β-hexosaminidase, α-galactosidase, α-mannosidase, arylsulfatase A and α-fucosidase enzyme activities, respectively. Plotted using Sigma plot 9.0 taking the averages of column values and standard deviation for the error bars.

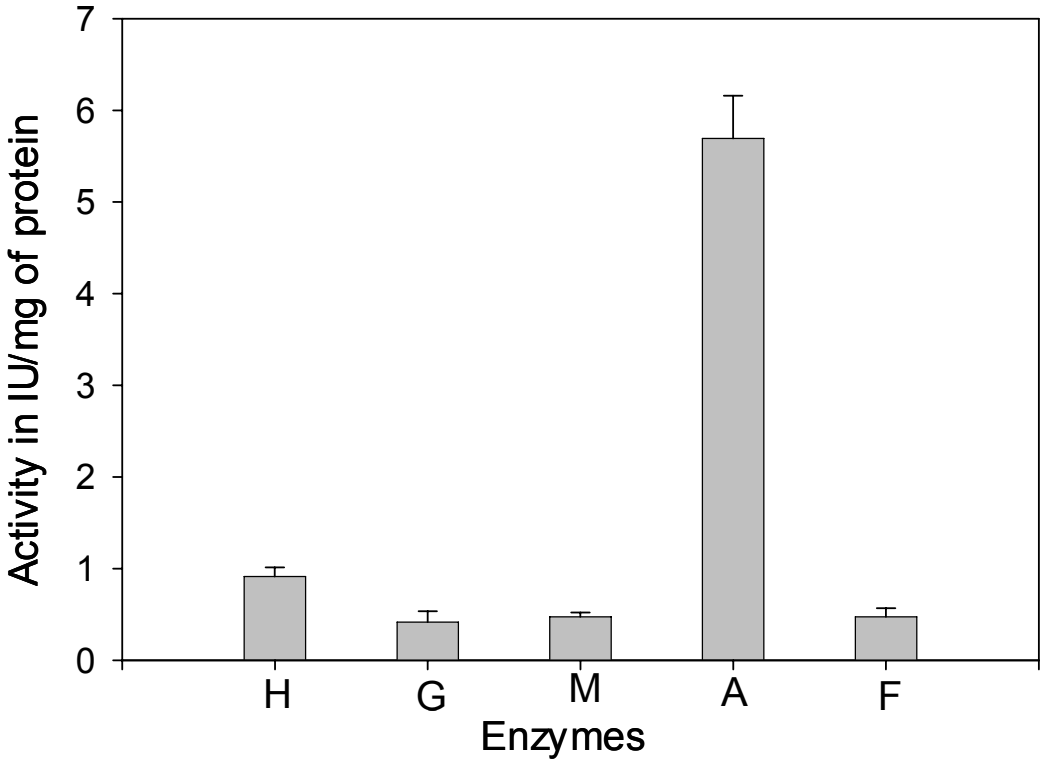


Figure 4A.4.1

Figure 4A.4.2: Immunoprecipitation (IP) of lysosomal enzymes and mannose 6-phosphate receptor proteins from [^{35}S] methionine labeled *Bg* cells with 250 µCi for 16 h, gels after fluorography and drying developed by autoradiography or exposed to phosphorimager. **(A) IP with arylsulfatase A antiserum**, both the precursor and mature forms are indicated by arrows; **(B) IP with β-hexosaminidase antiserum**, 'P' in the figure represents the precursor form of the enzyme; **(C) IP with α-fucosidase antiserum**, only one from is observed indicated by the arrow; **(D) IP with *unio* MPR 300 antiserum**; (**E) IP with MSC1 antibody**, arrow indicates the receptor protein, band indicated by '★' could be due to a cross-reactive material.

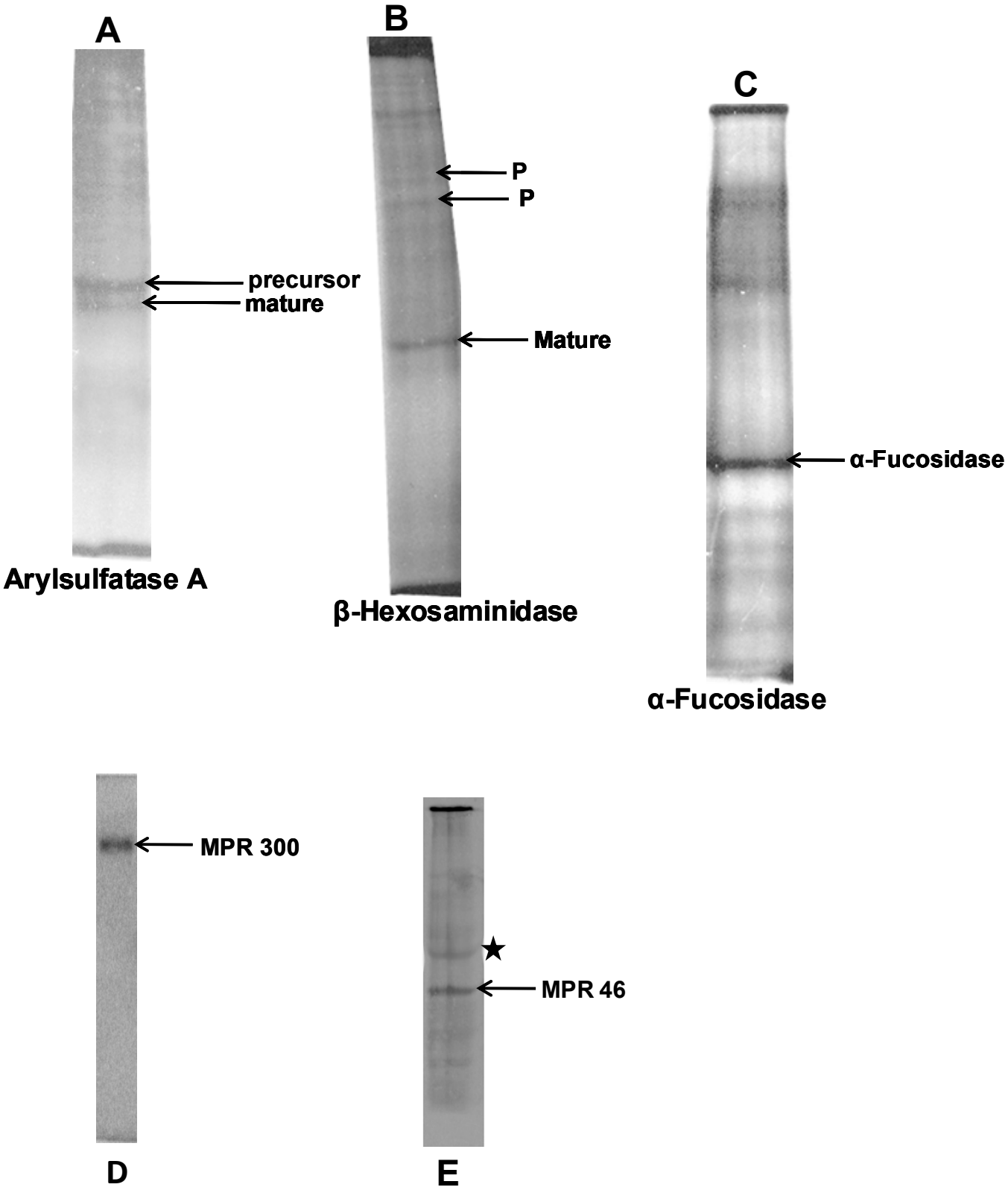

A
precursor
mature
Arylsulfatase A
B
P
P
Mature
β-Hexosaminidase
C
α-Fucosidase
α-Fucosidase
MPR 300
D
MPR 46
E

Figure 4A.4.2

Figure 4A.4.3: **Depletion of MPR 300 protein.** *Bg* cells pulsed with [^{35}S] methionine for 16 h and chased for MPR 300 protein using *unio* MPR 300 antiserum by IP. **(A)** chase up to 24 h from *Bg* cells treated with 1% preimmuneserum, the graph plotted below by taking the band intensities shows the disappearance of MPR 300 with a half life of ~ 13 h. **(B)** chase up to 240 min from *Bg* cells treated with 1% antiserum, the graph plotted below by taking the band intensities shows the drastic loss of MPR 300 with a half life of ~ 20 min.

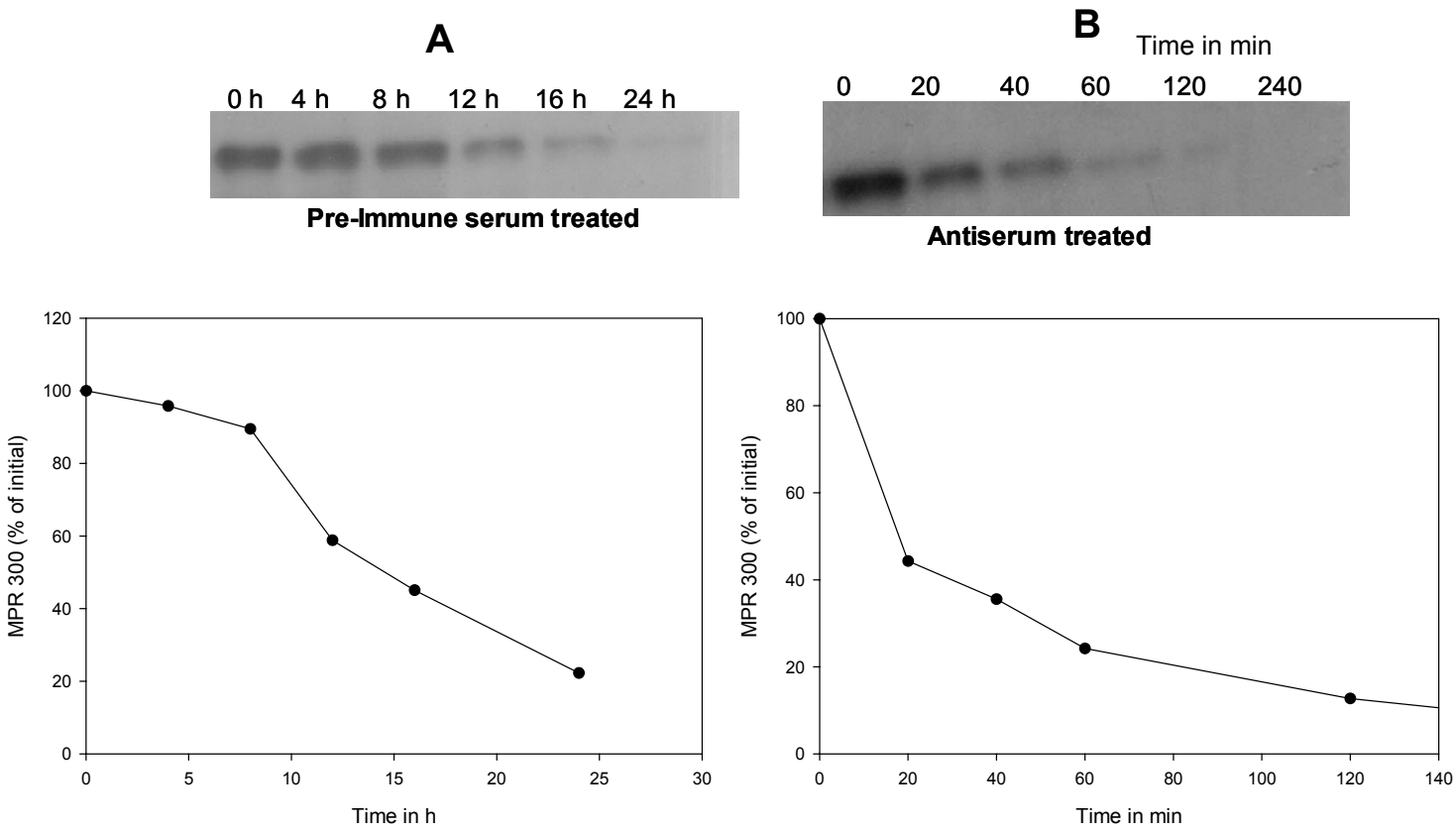

A
0 h 4 h 8 h 12 h 16 h 24 h
Pre-Immune serum treated
B
Time in min
0 20 40 60 120 240
Antiserum treated
MPR 300 (% of initial)
Time in h
MPR 300 (% of initial)
Time in min

Figure 4A.4.3

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

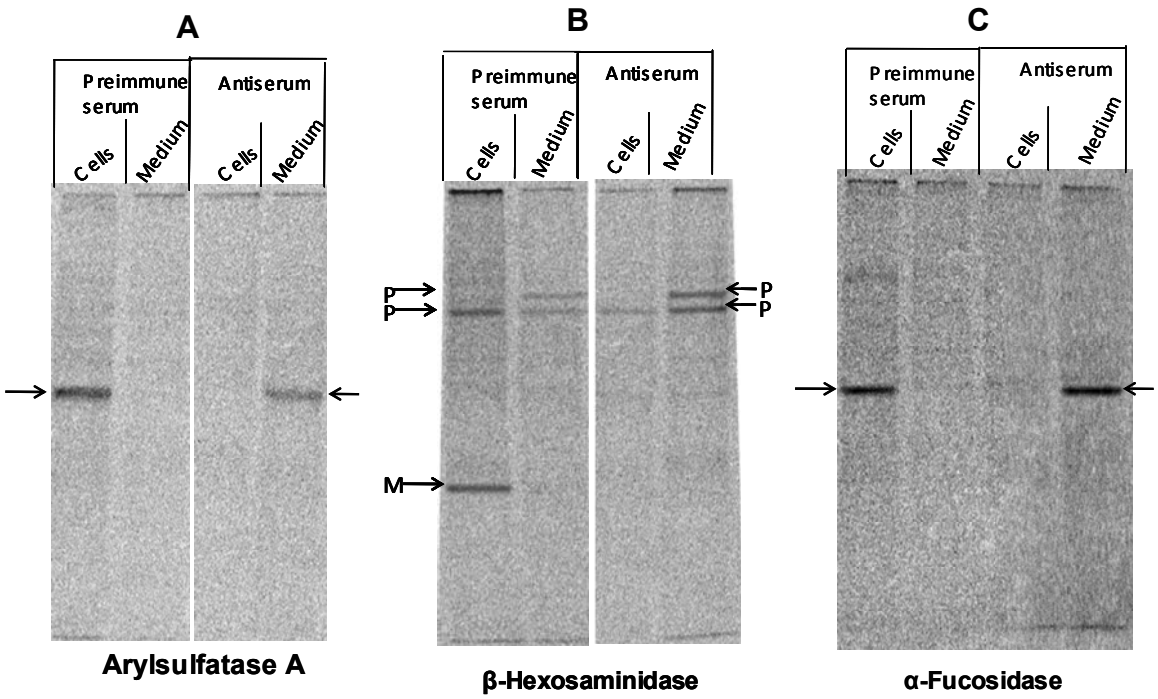

A
Preimmune serum
Antiserum
Cells
Medium
Cells
Medium
Arylsulfatase A
B
Preimmune serum
Antiserum
Cells
Medium
Cells
Medium
P
P
M
P
P
β-Hexosaminidase
C
Preimmune serum
Antiserum
Cells
Medium
Cells
Medium
α-Fucosidase

Figure 4A.4.4

Figure 4A.4.5: **Internalization of labeled lysosomal enzyme precursors**. The *Bg* cell lysate was analyzed for the presence of internalized labeled enzymes after an incubation of 24 h. Detected by IP with respective antiserum, arylsulfatase A (A), β-hexosaminidase (B) P in the figure represents the precursor form, and α-fucosidase (C).

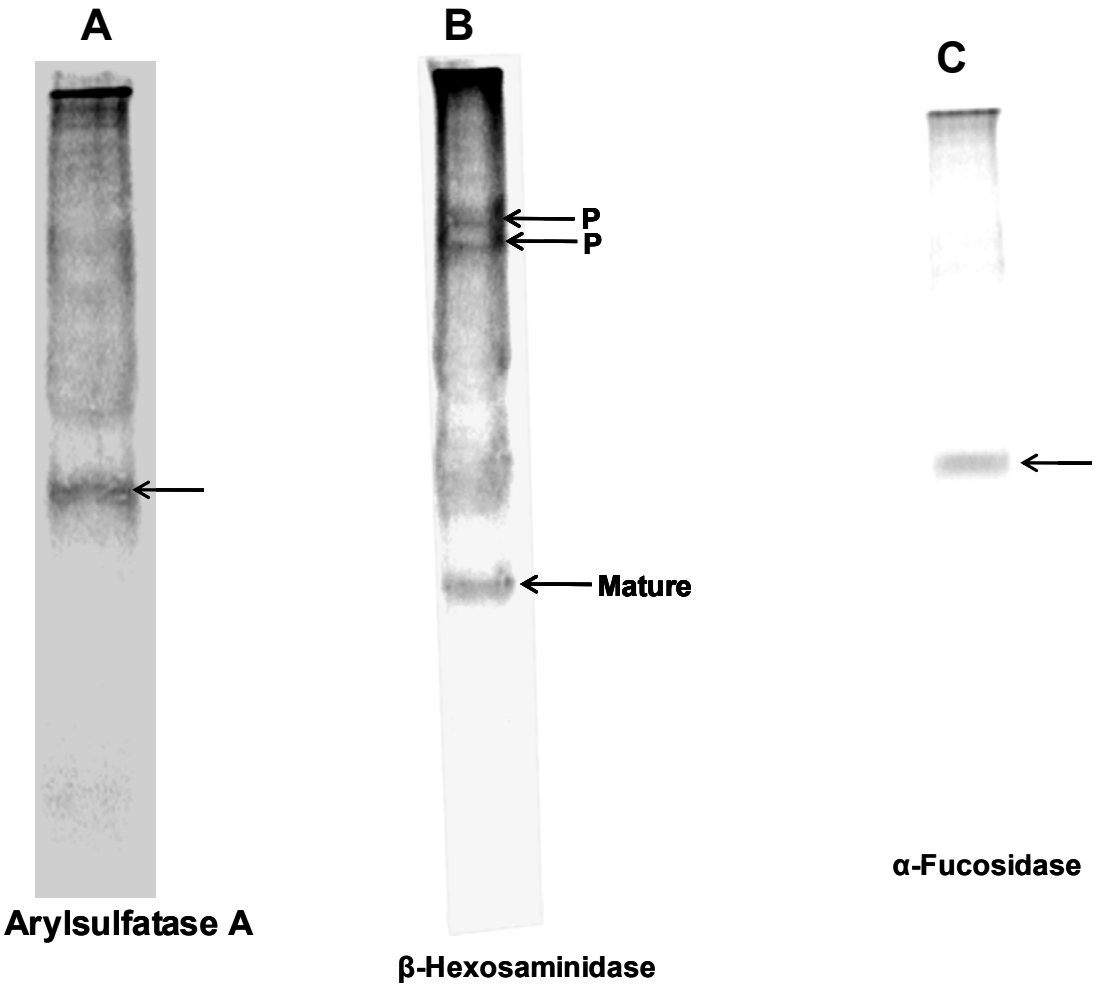

A
B
C
P
P
Mature
Arylsulfatase A
β-Hexosaminidase
α-Fucosidase

Figure 4A.4.5

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

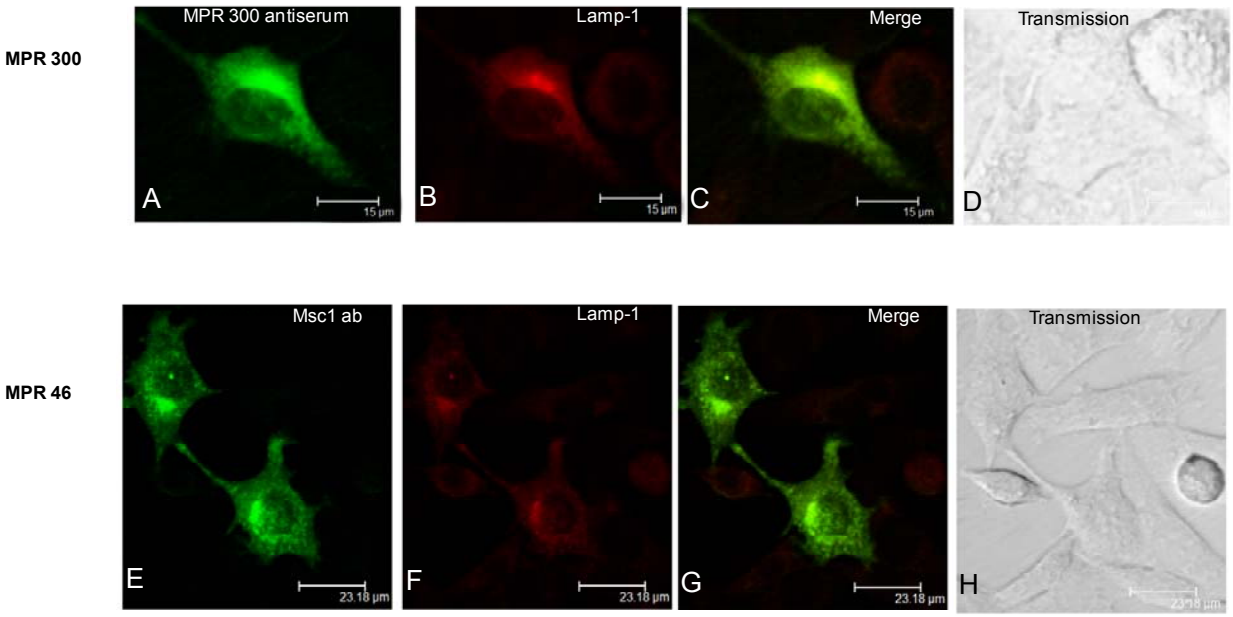

MPR 300
MPR 300 antiserum
Lamp-1
Merge
Transmission
A
B
C
D
15 µm
MPR 46
Msc1 ab
Lamp-1
Merge
Transmission
E
F
G
H
23.18 µm

Figure 4A.4.6

4A.5. DISCUSSION

We have earlier demonstrated the presence of MPR 46 and MPR 300 proteins in *unio* tissue as well in *Bg* cells by metabolic labeling and immunoprecipitation with MSC1 antibody and *unio* MPR 300 antiserum, respectively (Udaya Lakshmi et al., 1999; Siva Kumar and von Figura, 2002). Both the receptor proteins were purified to homogeneity by phosphomannan (PM) affinity chromatography, the study also confirmed the mannose 6-phosphate (M6P) dependent binding to PM gel. In this study we focused on the functional aspect of the receptor protein (MPR 300), which is to bind the bulk of lysosomal enzymes in a M6P dependent manner and target them to lysosomes. The MPR 300 protein also binds the extracellular ligands and internalizes them routing to lysosomes. We have identified lysosomal enzymes in *Bg* cells by enzyme assays and by immunoprecipitation of the [^{35}S] methionine labeled cellular proteins. Due to the limited availability of antibodies in our laboratory, we concentrated only on three lysosomal enzymes *viz.,* arylsulfatase A, β-hexosaminidase and α-fucosidase. The immunoprecipitated samples showed the existence of both the precursor and the mature forms. The antibody reactivity and presence of the receptors were confirmed by immunoprecipitation of MPR 300 and MPR 46 proteins using *unio* MPR 300 antiserum and MSC1 antibody, respectively. Our aim is to deplete the MPR 300 protein from *Bg* cells with the use of antiserum and monitor the relative distribution of lysosomal enzymes between cell lysate and medium. The MPR 300 receptor depletion was confirmed by its disappearance from the membranes with a half life of ~ 20 min (Fig 4A.4.3B). The antibodies present in the medium bind to MPR 300 present at the cell surface and makes it inactive further leading to internalization. This receptor-antibody complex upon internalization will lead to its degradation in lysosomes (these complexes that might have formed will escape the detection as the lysates were initially treated with protein A-agarose before immunoprecipitation). The dramatic loss of the receptor indicates that the cell surface receptor will always be in rapid equilibrium with that of intracellular receptor population. The loss of intracellular receptor explains the failure in sorting of newly synthesized lysosomal enzymes, and are hence secreted into the medium (about 80-85%), which are exclusively the precursor forms. The presence of 15-20% of enzymes in the cells treated with MPR 300 antiserum can be attributed to

MPR 46 function. The conversion of larger precursor forms in to smaller mature forms is thought to occur after their delivery to lysosomes (Hasilik and Neufeld, 1980; Waheed et al., 1982). In the preimmune serum treated cells very less or no enzyme was detected in the medium conversely bulk of the enzymes was seen intracellularly, and this observation is opposite in case of the antiserum treated cells. This establishes the functional role of the MPR 300 receptor protein in sorting of newly synthesized lysosomal enzymes. To further our knowledge on the endocytic function of the MPR 300 protein, we have incubated the *Bg* cells with NH_4Cl induced secretions from a different experiment with *Bg* cells. We then identified the internalized arylsulfatase A, β-hexosaminidase and α-fucosidase enzymes by immunoprecipitation. This suggests and signifies the importance of the cell surface MPR 300 in these cell types which is being able to internalize lysosomal enzymes and target them to the lysosomes. In a separate study we have seen the inhibition of uptake of radioiodinated β-galactosidase (from bovine) and α-fucosidase (from *unio)* in the presence of *unio* MPR 300 antiserum (data presented in continuation of this chapter PART-4B), which supports that the uptake of lysosomal enzymes is MPR 300 protein dependent. The immunofluorescence study shows the distribution of the receptor proteins to the endocytic structures. Lamp-1 is used as counter stain, only partial colocalization is observed as Lamp-1 is present on the late endosomes and lysosomes where the receptor proteins are present in very low amounts. The receptor protein and its bound lysosomal enzymes will dissociate in the early lysosomal compartment due to the acidic nature and recycle back to the *trans*-golgi network (TGN) or move to the plasma membrane to involve in endocytic process (kornfeld, 1992).

The role of the receptors in lysosomal enzyme targeting in the invertebrates is poorly understood. Only recently we have cloned the gene for the MPR 46 protein in the highly evolved invertebrate starfish and provided the first biochemical evidence to show that this receptor is involved in the lysosomal enzyme targeting, although no such studies have been done for the MPR 300 protein so far (Sivaramakrishna and Siva Kumar, 2008). The present study is an extension in this prospect, to further understand the lysosomal enzyme targeting system in the invertebrate systems. Since we have already identified and characterized the proteins in the molluscs, (*unio* and *Bg* cells), we

chose *Bg* cells to study the function of the MPR 300 protein. In summary this is the first report detailing the role of MPR 300 protein from *Bg* cells in segregating newly synthesized lysosomal enzymes and in the endocytosis of extracellular ligands. The findings support our earlier results and hypothesis that the MPR targeting system is an ancient pathway in evolution and it is conserved from molluscs to mammals. Below the molluscs, in annelids and arthropods we have observed only MPR 300 like proteins (Raju et al., 2001). In the *Drosophila melanogaster* lysosomal enzyme receptor protein (LERP) could be identified. Although this protein shows homology to the human MPR 300 to the extent of ~ 20%, it failed to bind the multivalent PM gel (Dennes et al., 2005), a characteristic feature exhibited by the MPR 300 protein from molluscs to mammals. It still needs to be established whether any other targeting system predates the existing well known trafficking system in the lower organisms below the molluscs in the animal kingdom.

In continuation of these findings, in our next section PART-4B, we studied the binding kinetics of MPR 300 from *Bg* cells towards β-galactosidase (from bovine) and α-fucosidase (from *unio)* in the presence and absence of specific and non-specific ligands.

PART B

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

4B.1. INTRODUCTION

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

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

4B.2. MATERIALS AND METHODS

4B.2.1. Materials

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

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

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

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

and about 12 fractions each of 0.5 ml were collected in 1.5 ml centrifuge tubes placed in the lead aluminum metal holder. 2 µl from each of the tube was aliquoted aside and counted for 1 min in a γ-ray counter.

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

The [^{125}I] β-galactosidase (~ 3 µCi/µg) was taken in a final concentration ranging from 5-50 nM. *Bg* cells grown to 80-85% confluency in 12 well culture plates were incubated with 10 different concentrations of [^{125}I] β-galactosidase for 90 min at 4^{o}C in binding buffer (Tris-HCl buffer pH 7.4, containing 0.1% BSA, 150 mM NaCl and 5 mM $CaCl_2$). In a separate experiment non-specific binding was determined in the presence of 2 µM non-radioactive β-galactosidase. After incubation, the cells were washed with PBS (10 mM sodium phosphate buffer pH 7.4, containing 150 mM NaCl) containing 1% BSA five times, lysed with lysis buffer (50 mM Tis-HCl buffer pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3 supplemented with PMSF, 1 mM EDTA and protease inhibitors) on ice for 30 min. The cell suspension was centrifuged at 2991xg for 2 min. The supernatant was discarded and pellets were counted in a gamma counter, and the amount of bound [^{125}I] β-galactosidase was calculated and normalized to the concentration of the membrane protein. Analysis was performed in duplicates and the average values were presented. Saturation and scatchard plot analysis were carried out using the details in (www.graphpad.com)

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

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

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

For treatment with Endo H and PNGase F glycosidases, [^{125}I] β-galactosidase and [^{125}I] α-fucosidase (5, 00,000 cpm each) was denatured by boiling with SDS (Endo

H treatment) and 2 µl of NP-40 (PNGase F treatment) incubated for 12 h at 37^0C in incubation buffer according to manufactures protocol containing 10 micro units/µl Endo H and 20 micro units/µl PNGase F (Sigma) (pH 7.0). For phosphatase treatment [^{125}I] β-galactosidase and [^{125}I] α-fucosidase was incubated in 0.15 M NaCl and 0.01 M Tris-HCl buffer pH 8.0, 50 milli units/µl alkaline phosphatase (Sigma), for 1 h at 37^0C, and then diluted to 1 ml with *Bg* medium. Effect of these treatments on endocytosis and binding was studied.

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

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

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

The endocytosis of [^{125}I] β-galactosidase by *Bg* cells was carried out in the absence or presence of M6P (5 mM), G6P (5 mM), D-mannose (5 mM), *unio* MPR 300 antiserum (1%) and preimmune serum (1%). *Bg* cells were grown to 80-85% confluency in 6 well culture plates. The cells were rinsed with *Bg* medium containing 20 mM HEPES pH 7.4 to remove the residual medium. The cells were then incubated with ligands mentioned above for 30 min at 28^oC in 1 ml of serum-free medium. At the end of additional 30 min incubation with [^{125}I] β-galactosidase (2, 00,000 cpm), the cells were washed six times with serum-free medium, three times with *Bg* medium containing 1 mg/ml bovine serum albumin and five times with PBS. The volume of each wash was 1

ml. After washings the cells were detached from the surface by gushing with TBS (20 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl), cell pellet was collected by centrifugation at 2991×g for 10 min, lysed in about 50 µl of TBS containing 150 mM NaCl, 1 mM PMSF, 1 µg/ml leupeptin, 1% Triton x-100, 0.05% NP-40. Radioactivity in the lysate was measured in a gamma counter and later applied on a 10% SDS-PAGE, and the gel processed for autoradiography. The importance of glycosylation on the radioiodinated enzyme was studied by incubating the *Bg* cells with ALP, PNGaseF and EndoH treated [^{125}I] β-galactosidase and followed its internalization. A bar graph was plotted in terms of the % of counts compared with the incubation of [^{125}I] β-galactosidase alone taking average of counts in cpm done in triplicates.

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

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

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

Membrane extraction from the *Bg* cells was done as described (page 53, section 2.2.4), to this 2, 00,000 cpm of [^{125}I] β-galactosidase or [^{125}I] α-fucosidase, was added as the case may be and made up to 1 ml by adding 20 mM Hepes pH 7.4 buffer containing 300 mM NaCl, 4 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM PMSF, 2 µg/ml leupeptin. This was kept for rotation for overnight at 4^{o}C, and the receptors and bound ^{125}I ligands were immunoprecipitated with *unio* MPR 300 antiserum prebound to protein A-agarose beads. The beads were washed with sodium phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST) 6-7 times and the bound [^{125}I] β-galactosidase or [^{125}I] α-fucosidase as the case may be, was specifically eluted with 5 mM M6P by incubating for 1 h at 4^{o}C. In a separate experiment 5 mM G6P and 5 mM D-mannose (non-specific ligands) were used in the elution step. The eluates were run on a 10% SDS-PAGE under reducing conditions and protein bands detected by autoradiography.

4B.2.10. Confocal microscopy

About 5 mg of α-fucosidase was incubated with 550 µl borate buffer (50 mM, pH 9.0) and 100 µl fluorochrome solution of fluorescein-isothiocyanate (5 mg FITC dissolved in one ml of DMSO) overnight at 4^0C. Free FITC was removed by desalting using a Sephadex G-25 gel. The endocytosis of FITC conjugated α-fucosidase was studied by incubating with *Bg* cells grown on cover slips at 28^oC. Following incubation of about 30 min the cells were fixed in 4% paraformaldehyde, washed, permeabilised with 0.2% Triton X-100, after blocking, cells were incubated with *unio* MPR 300 antiserum and detected using Cy5 labeled secondary antibody (Praveen Kumar and Siva Kumar, 2008). In a separate experiment the cells were pre-incubated for 30 min with 5 mM M6P before adding FITC conjugated α-fucosidase.

4B.3. RESULTS

4B.3.1. Determination of K_d values for [^{125}I] β-galactosidase and [^{125}I] α-fucosidase

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

4B.3.2. Effect of enzymatic deglycosylation on endocytosis and binding of [^{125}I] β-galactosidase and [^{125}I] α-fucosidase by MPRs

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

4B.3.3. Endocytosis of [^{125}I] β-galactosidase and [^{125}I] α-fucosidase by MPR 300 is M6P dependent

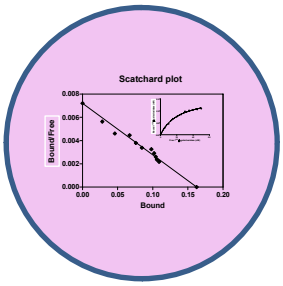
The endocytosis of [^{125}I] β-galactosidase and [^{125}I] α-fucosidase by *Bg* cells was carried out separately in the presence of 5 mM M6P, 5 mM G6P, 5 mM D-mannose, *unio* MPR 300 antiserum (1%) and preimmune serum (1%). After an incubation of 30 min with iodinated enzyme in the presence of these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

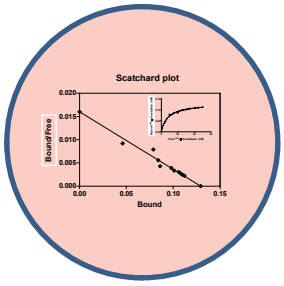
4B.3.4. Coimmunoprecipitation of MPR 300 and [^{125}I] β-galactosidase or [^{125}I] α-fucosidase

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

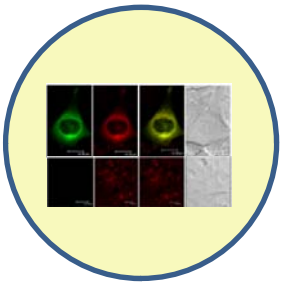
4B.3.5. Localization of endocytosed FITC-conjugated α-fucosidase

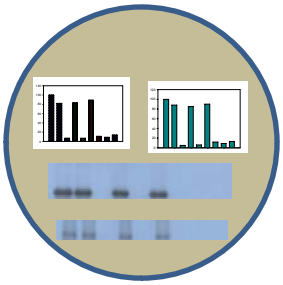
The endocytic function of the *Bg* cell MPR 300 protein was also revealed by confocal microscopy with the use of FITC-conjugated α-fucosidase. The endocytosed FITC-α-fucosidase can be seen in Fig 4B.4.6A (green). In the same cells, MPR 300 protein was also visualized with the use of *unio* MPR 300 antiserum and Cy5 labeled secondary antibody (Fig 4B.4.6B). Extensive colocalization of endocytosed FITC-α-fucosidase with the MPR 300 protein can be seen (Fig 4B.4.6C). The pre-incubation of *Bg* cells with 5 mM M6P resulted in failure of FITC-α-fucosidase uptake and the green fluorescence was not observed (Fig 4B.4.6E), the same cells were stained for the presence of MPR 300 protein (Fig 4B.4.6F).


Scatchard plot
Bound/Free
Bound


Scatchard plot
Bound/Free
Bound

FIGURES 4B.4





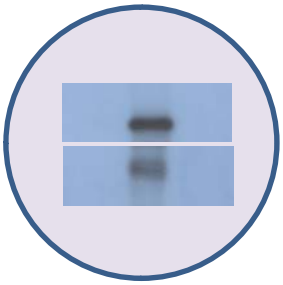


Figure 4B.4.1: **Saturation binding assay of [^{125}I] β-galactosidase on *Bg* cells**. The *Bg* cells were incubated with 10 different concentrations (5-50 nM) of the enzyme in a 12 well plate. The non-specific binding was determined by incubating the [^{125}I] β-galactosidase in presence of 2 μM non-radioactive β-galactosidase. The inset graph is the saturation curve plotted by taking free (on X-axis) verses specifically bound (on Y-axis) [^{125}I] β-galactosidase, and the scatchard plot analysis gave a K_d value of 22.52 nM. Graphs were plotted by graphpad prism 5.

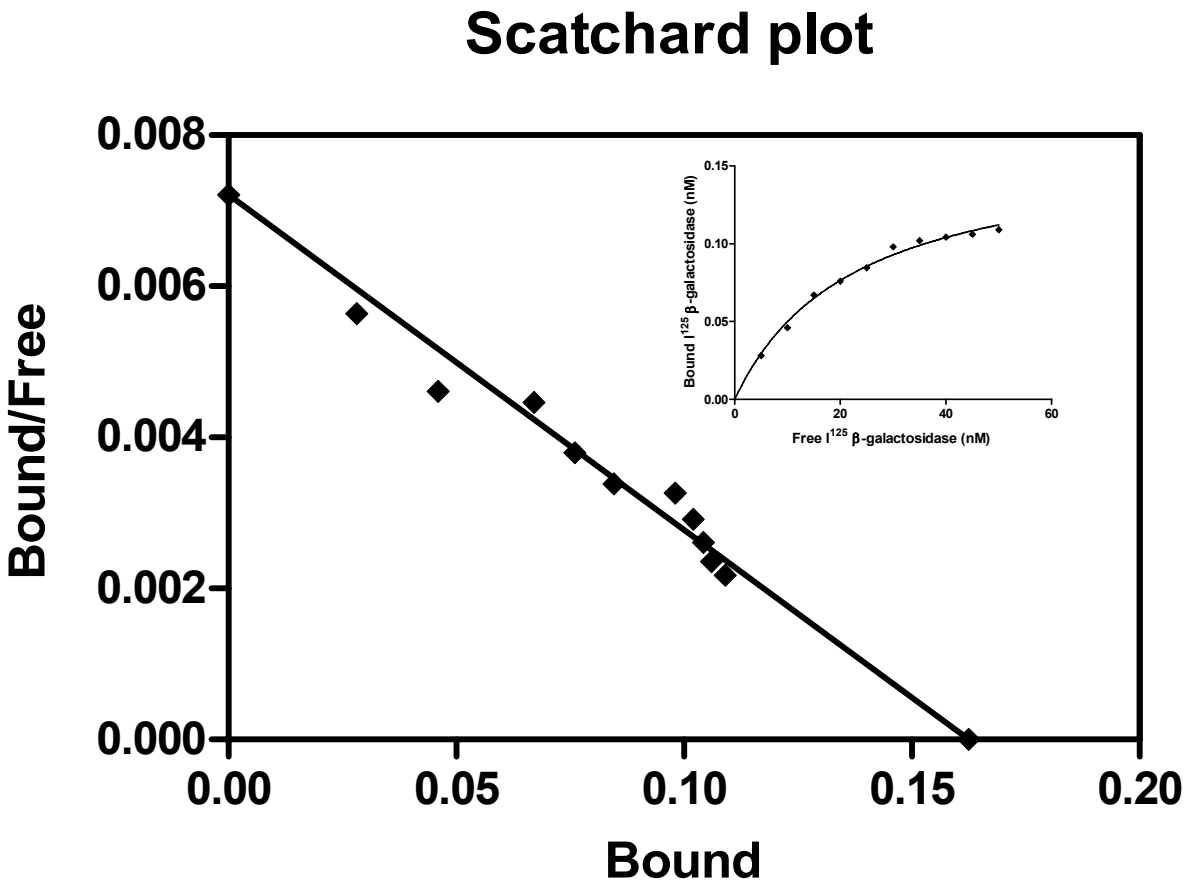

Scatchard plot
Bound/Free
0.008
0.006
0.004
0.002
0.000
0.00
0.05
0.10
0.15
0.20
Bound
Bound I125 β-galactosidase (nM)
0.15
0.10
0.05
0.00
0
20
40
60
Free I125 β-galactosidase (nM)

Figure 4B.4.1

Figure 4B.4.2: **Saturation binding assay of [^{125}I] α-fucosidase on *Bg* cells**. The *Bg* cells were incubated with 10 different concentrations (5-50 nM) of the enzyme in a 12 well plate. The non-specific binding was determined by incubating the [^{125}I] α-fucosidase in presence of 2 μM non-radioactive α-fucosidase. The graphs were plotted using graphpad prism 5. The inset is the saturation curve plotted by taking free (on X-axis) verses specifically bound (on Y-axis) [^{125}I] α-fucosidase, and the scatchard plot analysis taking bound (on X-axis) verses ratio of bound and free (on Y-axis) gave a K_d value of 8.090 nM.

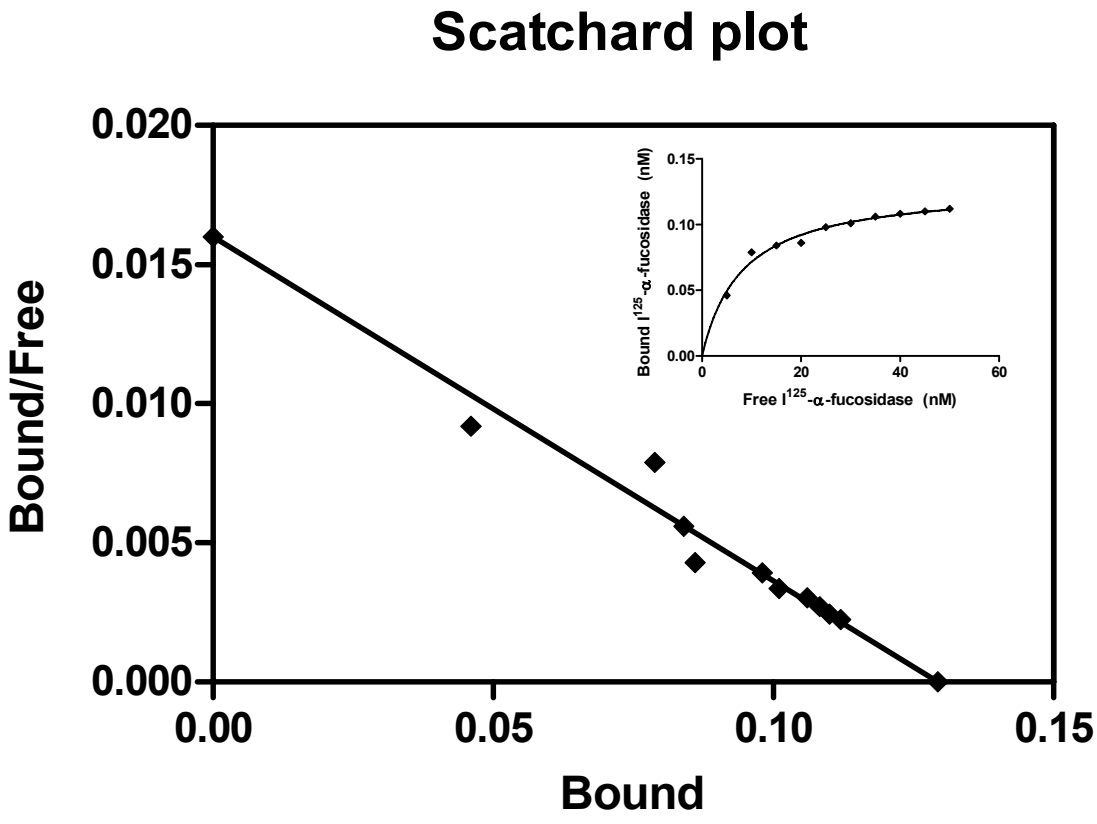
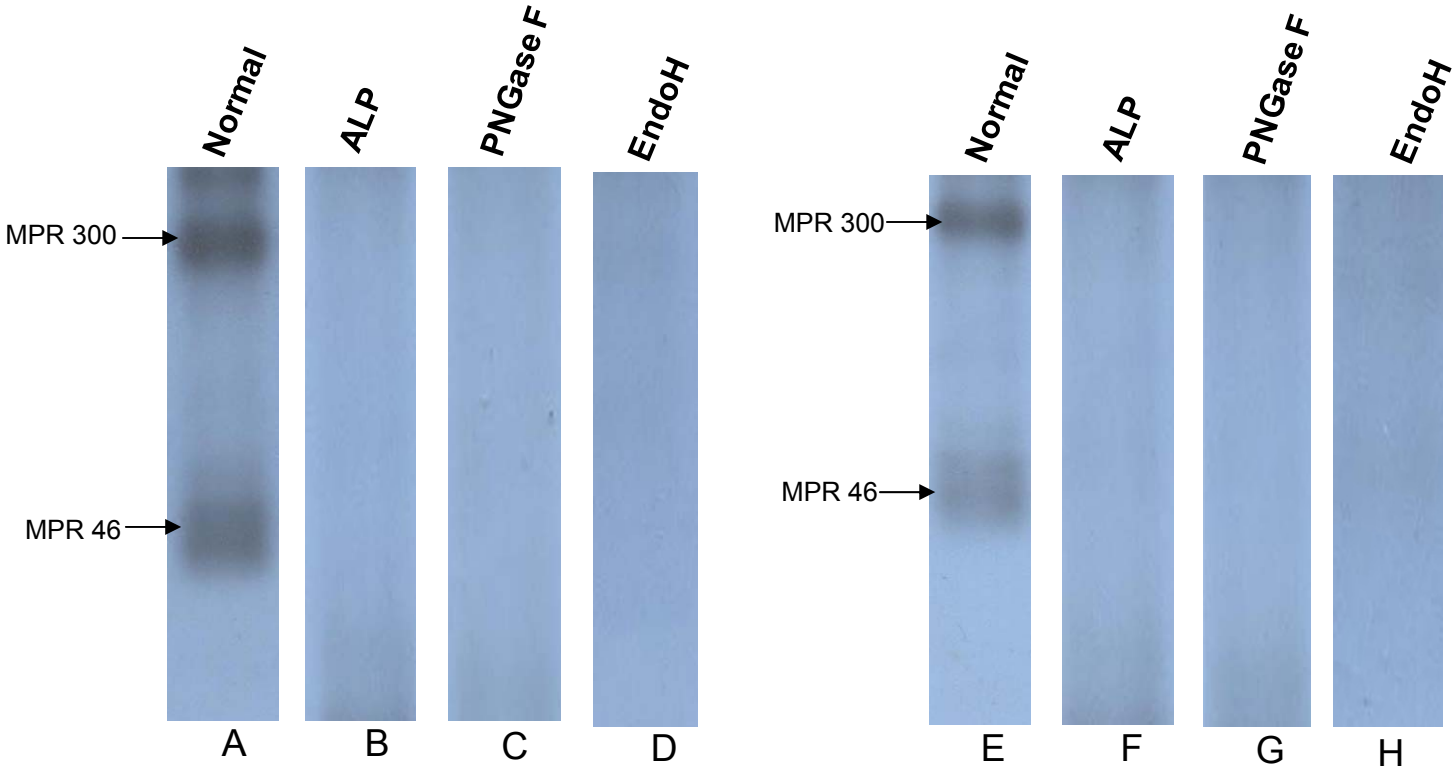

Scatchard plot
Bound/Free
0.020
0.015
0.010
0.005
0.000
0.00
0.05
0.10
0.15
Bound
Bound I125-α-fucosidase (nM)
0.15
0.10
0.05
0.00
0
20
40
60
Free I125-α-fucosidase (nM)

Figure 4B.4.2

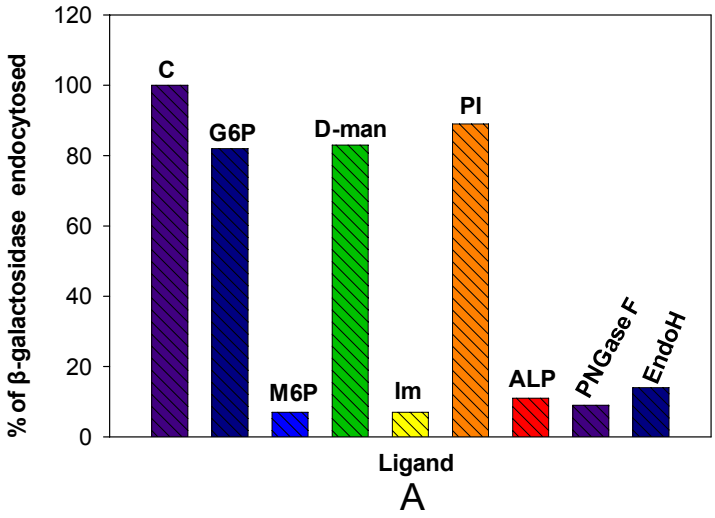
Figure 4B.4.3: **Ligand blotting**; about 35-40 µg of *Bg* cell membrane extract was separated on 10% SDS-PAGE and transferred to NC membrane. The cut lanes are incubated with 2, 00,000 cpm/ ml of [^{125}I] β-galactosidase; (A) normal, untreated. (B) ALP, alkaline phosphatase treated. (C) PNGase F, peptidyl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



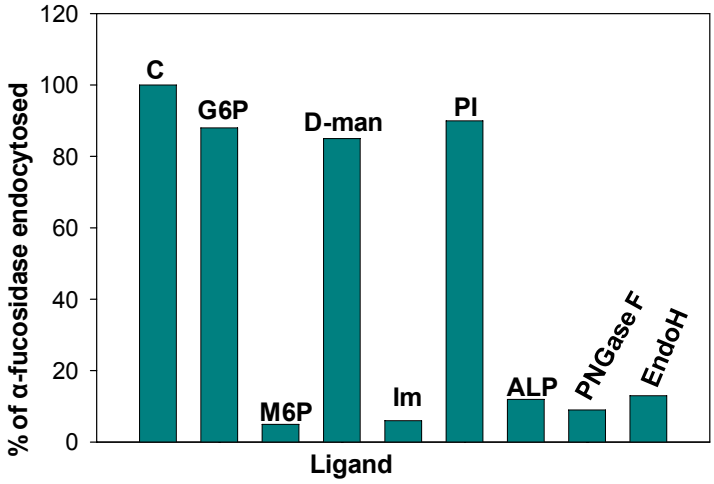
[^{125}I] β-galactosidase **[^{125}I] α-fucosidase**

Figure 4B.4.3

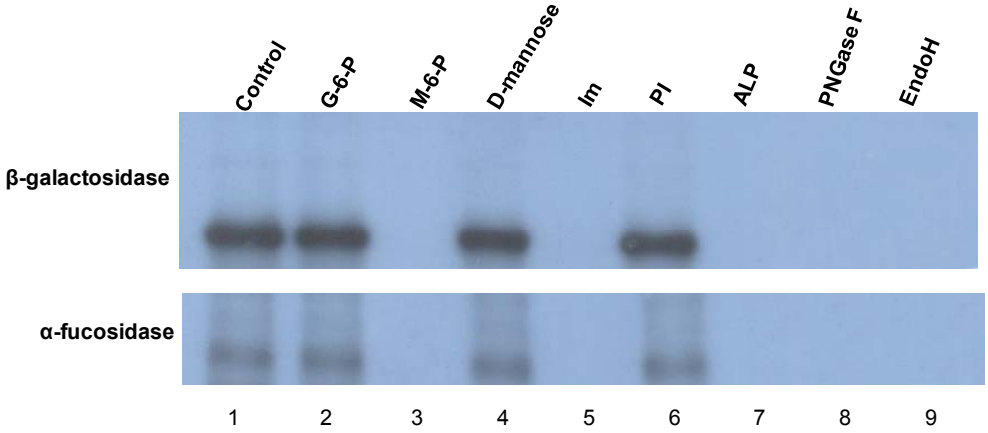
Figure 4B.4.4: Internalization of [^{125}I] β-galactosidase and [^{125}I] α-fucosidase by *Bg* cells. The cells were incubated with radioiodinated enzymes for 30 min at 28^{o}C with or without pre-incubation (30 min) of other ligands in serum free medium. **(A)** and **(B)** are the bar graphs plotted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



A



B



C

Figure 4B.4.4

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

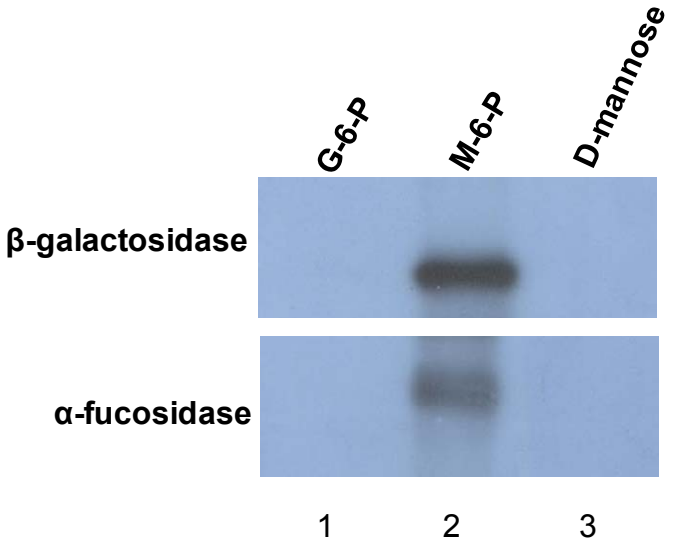


Figure 4B.4.5

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

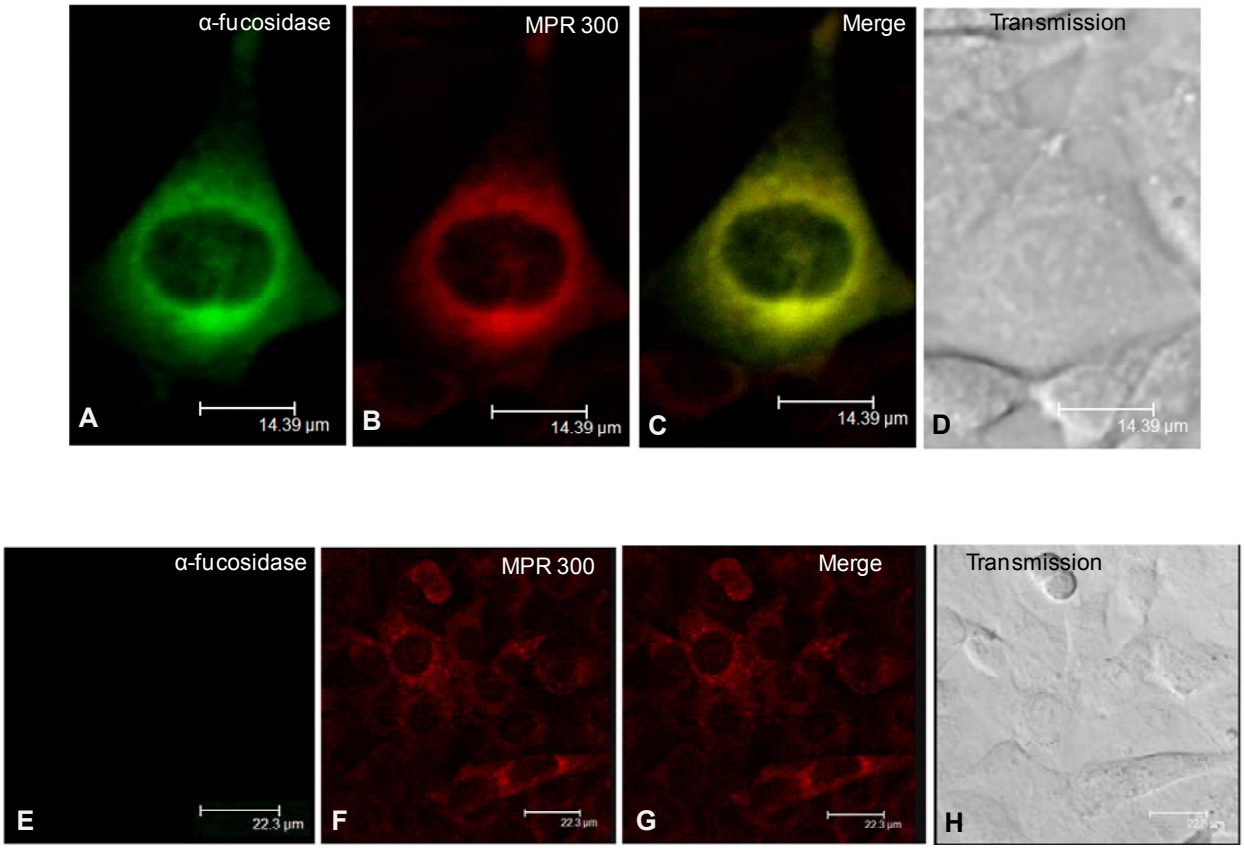

α-fucosidase
MPR 300
Merge
Transmission
A
14.39 µm
B
14.39 µm
C
14.39 µm
D
14.39 µm
α-fucosidase
MPR 300
Merge
Transmission
E
22.3 µm
F
22.3 µm
G
22.3 µm
H

Figure 4B.4.6

4B.5. DISCUSSION

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

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

internalized by the *Bg* cells, as only a limited internalization in the range of 11-12% was seen.

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

The sub cellular localization of the endocytosed enzyme was followed by using FITC-conjugated α-fucosidase and observing under confocal microscope. Here we have chosen α-fucosidase as it was from the same species and moreover it exhibited higher affinity to MPR 300 in the binding experiments. The MPR 300 protein is usually detected in endosomes, in the TGN, and at the plasma membrane in varying proportions in different cell types (Willingham et al., 1983; Geuze et al., 1984; Griffiths et al., 1988; Press et al., 1998). In earlier studies, the MPR 300 has been used as a marker for late

endosomes (Goda and Pfeffer, 1988; Griffiths et al., 1988). So the same cells which are incubated with FITC-α-fucosidase were fixed permeabilised and immunostained with *unio* MPR 300 antiserum to detect MPR 300 protein. The merged image of FITC-α-fucosidase (green) and MPR 300 protein (Cy5, red) showed extensive colocalization in the endocytic compartments. The localization of the FITC-α-fucosidase in early and late endosomes is well in agreement with our understanding of its trafficking to lysosomes by the MPR 300. Prior incubation of the cells with 5 mM M6P did not result in internalization of FITC-α-fucosidase and no green fluorescence was seen in the cells.

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

BIBLIOGRAHY

praveen

- **Admiraal PJ**, van Kesteren CA, Danser AH, Derkx FH, Sluiter W and Schalekamp MA. **(1999)** Uptake and proteolytic activation of prorenin by cultured human endothelial cells. *J. Hypertens*. 17; 621–629.

- **Aeed PA**, Guido DM, Mathews WR and Elhammer AP. **(1992)** Characterization of the oligosaccharide structures on recombinant human prorenin expressed in Chinese hamster ovary cells. *Biochemistry* 31; 6951–6961.

- **Alconada A**, Bauer U and Hoflack B. **(1996)** A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network. *EMBO J.* 15; 6096–6110.

- **Alexandrides T**, Moses AC and Smith RJ. **(1989)** Developmental expression of receptors for insulin, insulin-like growth factor I (IGF-I), and IGFII in rat skeletal muscle. *Endocrinology* 124; 1064–1076.

- **Appelmans F** and de Duve C. **(1955)** Tissue fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. *Biochem. J.* 59(3); 426–433.

- **Babst M**, Odorizzi G, Estepa EJ and Emr SD. (2000) Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. *Traffic.* 1; 248–258.

- **Bach G**, Bargal R and Cantz M. **(1979)** I-cell disease: deficiency of extracellular hydrolase phosphorylation. *Biochem. Biophys. Res. Commun.* 91; 976–981.

- **Barlow DP**, Stoger R, Herrmann BG, Saito K and Schweifer N. **(1991)** The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* 349; 84–87.

- **Bergman LW** and Kuehl WM. **(1978)**. Temporal relationship of translation and glycosylation of immunoglobulin heavy and light chains. *Biochemistry* 17; 5174–5180.

- **Beron W**, Alvarez-Dominguez C, Mayorga L and Stahl PD. (1995) Membrane trafficking along the phagocytic pathway. *Trends cell Biol.* 5; 100–103.

- **Berthe ML**, Esslimani Sahla M, Roger P, Gleizes M, Lemamy GJ, Brouillet JP and Rochefort H. **(2003)**. Mannose-6-phosphate/insulin-like growth factor-II receptor expression levels during the progression from normal human mammary tissue to invasive breast carcinomas. *Eur. J. Cancer*. 39; 635–642.

- **Blanchard F**, Duplomb L, Raher S, Vusio P, Hoflack B, Jacques Y and Godard A. **(1999)** Mannose 6-phosphate/insulin-like growth factor II receptor mediates internalization and degradation of leukemia inhibitory factor but not signal transduction. *J. Biol. Chem.* 274; 24685–24693.

- **Blanchard F**, Raher S, Duplomb L, Vusio P, Pitard V, Taupin JL, Moreau JF, Hoflack B, Minvielle S, Jacques Y and Godard A. **(1998)** The mannose 6-phosphate/insulin-like growth factor II receptor is a nanomolar affinity receptor for glycosylated human leukemia inhibitory factor. *J. Biol. Chem.* 273; 20886–20893.

- **Blum H,** Beier H and Gross HJ. **(1987)** Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis.* 8; 93–99

- **Bobek G**, Scott CD and Baxter RC. **(1992)** Radioimmunoassay of soluble insulin-like growth factor-II/mannose 6-phosphate receptor: developmental regulation of receptor release by rat tissues in culture. *Endocrinology* 130; 3387–3394.

- **Bonifacino JS** and Rojas R. **(2006)** Retrograde transport from endosomes to the trans-Golgi network. *Nat. Rev. Mol. Cell Biol.* 7; 568–579.

- **Brauker JH**, Roff CF and Wang JL. **(1986)** The effect of mannose 6-phosphate on the turnover of the proteoglycans in the extracellular matrix of human fibroblasts. *Exp. Cell Res.* 164; 115–126.

- **Braulke T**. **(1999)** Type-2 IGF receptor: a multiple-ligand binding protein. *Horm. Metab. Res.* 31; 242–246.

- **Braulke T**, Bresciani R, Buergisser DM and von Figura K. **(1991)** Insulin-like growth factor II overexpression does not affect sorting of lysosomal enzymes in NIH-3T3 cells. *Biochem. Biophys. Res. Commun.* 179(1); 108–115.

- **Braulke T**, Gartung C, Hasilik A and von Figura K. **(1987)** Is movement of mannose 6-phosphate-specific receptor triggered by binding of lysosomal enzymes? *J. Cell Biol.* 104; 1735–1742.

- **Braulke T** and Mieskes G. **(1992)** Role of protein phosphatases in insulin-like growth factor II (IGF II)-stimulated mannose 6-phosphate/IGF II receptor redistribution. *J. Biol. Chem.* 267; 17347–17353.

- **Braulke T**, Tippmer S, Chao HJ and von Figura K. **(1990)** Insulin-like growth factors I and II stimulate endocytosis but do not affect sorting of lysosomal enzymes in human fibroblasts. *J. Biol. Chem.* 265; 6650–6655.

- **Braulke T**, Tippmer S, Neher E and von Figura K. **(1989)** Regulation of the mannose 6-phosphate/IGF II receptor expression at the cell surface by mannose 6-phosphate, insulin like growth factors and epidermal growth factor, *EMBO J.* 8; 681–686.

- **Breuer P** and Braulke T. **(1998)** Stabilization of mutant 46-kDa mannose 6-phosphate receptors by proteosomal inhibitor lactacystin. *J. Biol. Chem.* 273; 33254–33258.

- **Breuer P**, Korner C, Boker C, Herzog A, Pohlmann R. and Braulke T. **(1997)** Serine phosphorylation site of the 46-kDa mannose 6-phosphate receptor is required for transport to the plasma membrane in Madin – Darby canine kidney and mouse fibroblast cells. *Mol. Biol. Cell* 8; 567–576.

- **Brown J**, Esnouf RM, Jones MA, Linnell J, Harlos K, Hassan AB and Jones EY. **(2002)** Structure of a functional IGF2R fragment determined from the anomalous scattering of sulfur. *EMBO J.* 21; 1054–1062.

- **Brunetti CR**, Burke RL, Hoflack B, Ludwig T, Dingwell KS and Johnson DC. **(1995)** Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. *J. Virol*. 69; 3517–3528.

- **Brunetti CR**, Burke RL, Kornfeld S, Gregory W, Masiarz FR, Dingwell KS and Johnson DC. **(1994)** Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. *J. Biol. Chem*. 269; 17067–17074.

- **Brunetti CR**, Dingwell KS, Wale C, Graham FL and Johnson DC. **(1998)** Herpes simplex virus gD and virions accumulate in endosomes by mannose 6-phosphate-dependent and -independent mechanisms. *J. Virol*. 72; 3330–3339.

- **Burkhardt JK**, Hester S and Argon Y. **(1989)** Two proteins targeted to the same lytic granule compartment undergo very different posttranslational processing. *Proc. Natl. Acad. Sci. U.S.A*. 86; 7128–7132.

- **Byrd JC**, Park JH, Schaffer BS, Garmroudi F and MacDonald RG. **(2000)** Dimerization of the insulin-like growth factor II/mannose 6-phosphate receptor. *J. Biol. Chem*. 275; 18647–18656.

- **Cacia J**, Quan CP, Pai R and Frenz J. **(1998)** Human DNase I contains mannose 6-phosphate and binds the cation-independent mannose 6-phosphate receptor. *Biochemistry* 37; 15154–15161.

- **Canfield WM**, Johnson KF, Ye RD, Gregory W and Kornfeld S. **(1991)** Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor to amino acids 24– 29 of the cytoplasmic tail. *J. Biol. Chem*. 266; 5682–5688.

- **Catherine MN**, McCarthy K, Eivers E, Jirtle RL and Byrnes L. **(2006)** Mannose 6-phosphate receptors in an ancient vertebrate Zebra fish. *Dev. Genes. Evol*. 216; 144–151.

- **Chao HH**, Waheed A, Pohlmann R, Hille A and von Figura K. **(1990)** Mannose 6-phosphate receptor dependent secretion of lysosomal enzymes. *EMBO J*. 9; 3507–3513.

- **Chappell SA**, Walsh T, Walker RA and Shaw JA. **(1997)** Loss of heterozygosity at the mannose 6-phosphate insulin-like growth factor 2 receptor gene correlates with poor differentiation in early breast carcinomas. *Br. J. Cancer*. 76; 1558–1561.

- **Chen HJ**, Yuan J and Lobel P. **(1997)** Systematic mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain. An acidic cluster containing a key aspartate is important for function in lysosomal enzyme sorting. *J. Biol. Chem*. 272; 7003–7012.

- **Clairmont KB** and Czech MP. **(1989)** Chicken and Xenopus mannose 6-phosphate receptors fail to bind insulin-like growth factor II. *J. Biol. Chem*. 264; 16390–16392.

- **Costello M**, Baxter RC and Scott CD. **(1999)** Regulation of soluble insulinlike growth factor II/mannose 6-phosphate receptor in human serum: measurement by enzyme-linked immunosorbent assay. *J. Clin. Endocrinol. Metab*. 84; 611–617.

- **Creek KE** and Sly WS. **(1983)** Biosynthesis and turnover of the phosphomannosyl receptor in human fibroblasts. *Biochem J.* 214(2); 353–360.

- **Crump CM**, Xiang Y, Thomas L, Gu F, Austin C, Tooze SA and Thomas G. **(2001)** PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic. *EMBO J.* 20; 2191–2201.

- **Dahms NM**, Brzycki-Wessell MA, Ramanujam KS and Seetharam B. **(1993)** Characterization of mannose 6-phosphate receptors (MPRs) from opossum liver: opossum cation-independent MPR binds insulin-like growth factor-II. *Endocrinology* 133; 440–446.

- **Dahms NM** and Hancock MK. **(2002)** P-type lectins. *Biochim. Biophys. Acta* 1572; 317–340.

- **Dahms NM** and Kornfeld S. **(1989)** The cation-dependent mannose 6-phosphate receptor. Structural requirements for mannose 6-phosphate binding and oligomerization. *J. Biol. Chem*. 264; 11458–11467.

- **Dahms NM**, Lobel P, Breitmeyer J, Chirgwin JM and Kornfeld S. **(1987)** 46 kDa mannose 6-phosphate receptor: cloning, expression and homology to the 215 kDa mannose 6-phosphate receptor. *Cell* 50, 181–192.

- **Dahms NM**, Rose PA, Molkentin JD, Zhang Y and Brzycki MA. **(1993)** The bovine mannose 6-phosphate/insulin-like growth factor II receptor. The role of arginine residues in mannose 6-phosphate binding. *J. Biol. Chem*. 268; 5457–5463.

- **Dahms NM**, Wick DA and Brzycki-Wessell MA. **(1994)** The bovine mannose 6-phosphate/insulin-like growth factor II receptor. Localization of the insulin-like growth factor II binding site to domains 5 –11. *J. Biol. Chem*. 269; 3802–3809.

- **Damke H**, von Figura K and Braulke T. **(1992)** Simultaneous redistribution of mannose 6-phosphate and transferrin receptors by insulin-like growth factors and phorbol ester. *Biochem. J*. 281; 225–229.

- **Danser AH**, Saris JJ, Schuijt MP and van Kats JP. **(1999)** Is there a local renin-angiotensin system in the heart? *Cardiovasc. Res*. 44; 252–265.

- **de Duve C** and Wattiaux R. **(1966)** Functions of lysosomes. *Annu.Rev.Physiol*. 28;435–492.

- **Dell'Angelica EC** and Payne GS. **(2001)** Intracellular cycling of lysosomal enzyme receptors. Cytoplasmic tails' tales. *Cell* 106; 395–398.

- **Dennes A**, Cromme C, Koduru S, Siva Kumar N, Eble JA, Hahnenkamp A and 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.

- **Dennis PA** and Rifkin DB. **(1991)** Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc. Natl. Acad. Sci. U.S.A*. 88; 580–584.

- **Denzer K**, Weber B, Hille-Rehfeld A, Figura KV and Pohlmann R. **(1997)** Identification of three internalization sequences in the cytoplasmic tail of the 46 kDa mannose 6-phosphate receptor. *Biochem. J*. 326; 497–505.

- **De Souza AT**, Hankins GR, Washington MK, Fine RL, Orton TC and Jirtle RL. **(1995)** Frequent loss of heterozygosity on 6q at the mannose 6-phosphate/insulin-like growth factor II receptor locus in human hepatocellular tumors. *Oncogene.* 10; 1725–1729.

- **Devi GR**, Byrd JC, Slentz DH and MacDonald RG. **(1998)** An insulin-like growth factor II (IGF-II) affinity-enhancing domain localized within extracytoplasmic repeat 13 of the IGF-II/mannose 6-phosphate receptor. *Mol. Endocrinol*. 12; 1661–1672.

- **Diaz E** and Pfeffer SR. **(1998)** TIP47: a cargo selection device for mannose-6-phosphate receptor trafficking. *Cell* 93; 433–443.

- **Distler JJ**, Guo JF, Jourdian GW, Srivastava OP and Hindsgaul O. **(1991)** The binding specificity of high and low molecular weight phosphomannosyl receptors from bovine testes. Inhibition studies with chemically synthesized 6-O-phosphorylated oligomannosides. *J. Biol. Chem*. 266; 21687–21692.

- **Distler J,** Hieber V, Sahagian G, Schmickel R and Jourdian GW. **(1979)** Identification of Manose 6-Phosphate in Glycoproteins that Inhibit the Assimiation of Galactosidase by Fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 76; 4235–4239.

- **Drickamer K** and Taylor ME. **(1993)** Biology of animal lectins. *Annu. Rev.Cell Biol*. 9; 237–264.

- **Dittmer F**, Hafner A, Ulbrich EJ, Moritz JD, Schmidt P, Schmahl W, Pohlmann R and von Figura K. **(1998)** I-cell disease-like phenotype in mice deficient in mannose 6-phosphate receptors. *Transgenic Res*. 7; 473–483.

- **Faust PL**, Chirgwin JM and Kornfeld S. **(1987)** Renin, a secretory glycoprotein, acquires phosphomannosyl residues. *J. Cell Biol*. 105; 1947–1955.

- **Frenz J**, Quan CP, Cacia J, Democko C, Bridenbaugh R and McNerney T. **(1994)** Protein sorting by high performance liquid chromatography: 2. Separation of isophosphorylates of recombinant human DNase I on a polyethylenimine column. *Anal. Chem*. 66; 335–340.

- **Funk B**, Kessler U, Eisenmenger W, Hansmann A, Kolb HJ and Kiess W. **(1992)** Expression of the insulin-like growth factor-II/mannose-6-phosphate receptor in multiple human tissues during fetal life and early infancy. *J. Clin. Endocrinol. Metab*. 75; 424–431.

- **Gabel CA**, Dubey L, Steinberg SP, Sherman D, Gershon MD and Gershon AA. **(1989)** Varicella-zoster virus glycoprotein oligosaccharides are phosphorylated during posttranslational maturation. *J. Virol*. 63; 4264–4276.

- **Gabel CA**, Goldberg DE and Kornfeld S. **(1983)** Identification and characterization of cells deficient in the mannose 6-phosphate receptor: evidence for an alternate pathway for lysosomal enzyme targeting. *Proc. Natl. Acad. Sci. U.S.A*. 80; 775–779.

- **Garmroudi F**, Devi G, Slentz DH, Schaffer BS and MacDonald RG. **(1996)** Truncated forms of the insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor encompassing the IGF-II binding site: characterization of a point mutation that abolishes IGF-II binding. *Mol. Endocrinol*. 10; 642–651.

- **Garmroudi F** and MacDonald RG. **(1994)** Localization of the insulinlike growth factor II (IGF-II) binding/cross-linking site of the IGF-II/mannose 6-phosphate receptor to extracellular repeats 10–11. *J. Biol. Chem*. 269; 26944–26952.

- **Gartung C**, Braulke T, Hasilik A and Von Figura K. **(1985)** Internalization of blocking antibodies against mannose-6-phosphate specific receptors. *EMBO J.* 4; 1725–1730.

- **Gary-Bobo M**, Nirdé P, Jeanjean A, Morère A and Garcia M. **(2007)** Mannose 6-Phosphate Receptor Targeting and its Applications in Human Diseases. *Current Medicinal Chemistry.* 14; 2945–2953.

- **Gerweck LE**, Kozin SV and Stocks SJ. **(1999)** The pH partition theory predicts the accumulation and toxicity of doxorubicin in normal and low-pH-adapted cells. *Br. J. Cancer*. 79; 838–842.

- **Geuze HJ**, Slot JW, Strous GJAM, Hasilik A and von Figura K. **(1984)** Ultrastructural localization of the mannose 6-phosphate receptor in rat liver. *J. Cell Biol*. 98; 2047–2054.

- **Ghahary A**, Tredget EE, Shen Q, Kilani RT, Scott PG and Houle Y. **(2000)** Mannose-6-phosphate/IGF-II receptors mediate the effects of IGF-1-induced latent transforming growth factor beta 1 on expression of type I collagen and collagenase in dermal fibroblasts. *Growth Factors.* 17; 167–176.

- **Ghahary A**, Tredget EE and Shen Q. **(1999)** Insulin-like growth factor-II/ mannose 6 phosphate receptors facilitate the matrix effects of latent transforming growth factor-beta1 released from genetically modified keratinocytes in a fibroblast/keratinocyte co-culture system. *J. Cell Physiol*. 180; 61–70.

- **Ghosh P**, Dahms NM and Kornfeld S. **(2003)** Mannose 6-phosphate receptor: new twists in the tale. *Nat. Rev. Mol. Cell Biol*. 4; 202–212.

- **Glaser JH**, McAlister WH and Sly WS. **(1974)** Genetic heterogeneity in multiple lysosomal hydrolase deficiency. *J. Pediatr.* 85; 192–198.

- **Glickman JN**, Conibear E and Pearse BM. **(1989)** Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO J.* 8; 1041–1047.

- **Godar S**, Horejsi V, Weidle UH, Binder BR, Hansmann C and Stockinger H. **(1999)** M6P/IGFII-receptor complexes urokinase receptor and plasminogen for activation of transforming growth factor-beta1. *Eur. J. Immunol*. 29; 1004–1013.

- **Goda Y** and Pfeffer SR. **(1988)** Selective recycling of the mannose 6-phosphate/IGF-II receptor to the *trans*-Golgi network in vitro. *Cell* 55; 309–320.

- **Griffiths G**, Hoflack B, Simons K, Mellman I, and Kornfeld S. **(1988)** The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52; 329–341.

- **Griffiths GM** and Isaaz S. **(1993)** Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose-6-phosphate receptor. *J. Cell Biol*. 120; 885–896.

- **Groskopf JC**, Syu LJ, Saltiel AR and Linzer DI. **(1997)** Proliferin induces endothelial cell chemotaxis through a G protein-coupled, mitogenactivated protein kinase-dependent pathway. *Endocrinology* 138; 2835–2840.

- **Guruprasad RM** and Peter Schu **(2003)** Characterization of the in Vitro Retrograde Transport of MPR46. *Traffic.* 4; 802–811.

- **Han VK**, D'Ercole AJ and Lund PK. **(1987)** Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science* 236; 193-197.

- **Hancock MK**, Haskins DJ, Sun G and Dahms NM. **(2002)** Identification of residues essential for carbohydrate recognition by the insulin-like growth factor II/mannose 6-phosphate receptor. *J. Biol. Chem*. 277; 11255–11264.

- **Hankins GR,** De Souza .T, Bentley RC, Patel MR, Marks JR, Iglehart JD and Jirtle RL. **(1996)** M6P/IGF2 receptor: a candidate breast tumour suppressor gene. *Oncogene.* 12; 2003–2009.

- **Haslik A** and Neufeld EF. **(1980)** Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursors of higher molecular weight. *J. Biol. Chem*. 255**;** 4937–4945.

- **Hassan AB**. **(2003)** Keys to the hidden treasures of the mannose 6-phosphate/insulin-like growth factor 2 receptor. *Am. J. Pathol*. 162; 3–6.

- **Hemer F**, Korner C and Braulke T. **(1993)** Phosphorylation of the human 46-kDa mannose 6-phosphate receptor in the cytoplasmic domain at serine 56. *J. Biol. Chem*. 268; 17108–17113.

- **Herzog V**, Neumuller W and Holzmann B. **(1987)** Thyroglobulin, the major and obligatory exportable protein of thyroid follicle cells, carries the lysosomal recognition marker mannose-6-phosphate. *EMBO J*. 6; 555–560.

- **Hickman S** and Neufeld EF. **(1972)** A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem. Biophys. Res. Commun.* 49; 992–999.

- **Hickman S**, Shapiro LJ and Neufeld EF. **(1974)** A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. *Biochem. Biophys. Res. Commun.* 57; 55–61.

- **Hille-Rehfeld A**. **(1995)** Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim. Biophys. Acta.* 1241; 177–194.

- **Hille A**, Waheed A and von Figura K. **(1990)** Assembly of the ligand-binding conformation of Mr 46,000 mannose 6-phosphate-specific receptor takes place before reaching the Golgi complex. *J. Cell Biol*. 110; 963–972.

- **Hille A**, Waheed A and von Figura K. **(1989)** The ligand-binding conformation of Mr 46,000 mannose 6-phosphate-specific receptor. Acquisition of binding activity during in vitro synthesis. *J. Biol. Chem*. 264; 13460–13467.

- **Hoflack B** and Kornfeld S. **(1985)** Purification and characterization of a cationdependent mannose 6-phosphate receptor from murine P388D1 macrophages and bovine liver. *J. Biol. Chem*. 260; 12008–12014.

- **Hogg J**, Hill DJ and Han VK. **(1994)** The ontogeny of insulin-like growth factor (IGF) and IGF-binding protein gene expression in the rat pancreas. *J. Mol. Endocrinol*. 13; 49–58.

- **Honing S**, Sosa M, Hille-Rehfeld A and von Figura K. **(1997)** The 46-kDa mannose 6-phosphate receptor contains multiple binding sites for clathrin adaptors. *J. Biol. Chem*. 272; 19884–19890.

- **Hu KQ**, Backer JM, Sahagian GG, Feener EP and King GL. **(1990)** Modulation of the insulin growth factor II/mannose 6-phosphate receptor in microvascular endothelial cells by phorbol ester via protein kinase C. *J. Biol. Chem*. 265; 13864–13870.

- **Ikushima H**, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, Schlossman SF and Morimoto C. **(2000)** Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. *Proc. Natl. Acad. Sci. U.S.A*. 97; 8439–8444.

- **Itin C**, Rancano C, Nakajima Y and Pfeffer SR. **(1997)** A novel assay reveals a role for soluble N-ethylmaleimide-sensitive fusion attachment protein in mannose 6-phosphate receptor transport from endosomes to the trans Golgi network. *J. Biol. Chem*. 272; 27737–27744.

- **Itin C**, Ulitzur N, Muhlbauer B and Pfeffer SR. **(1999)** Mapmodulin, cytoplasmic dynein, and microtubules enhance the transport of mannose 6-phosphate receptors from endosomes to the trans-Golgi network. *Mol. Biol. Cell* 10; 2191–2197.

- **Jackson D** and Linzer DI. **(1997)** Proliferin transport and binding in the mouse fetus. *Endocrinology* 138; 149–155.

- **Jadot M**, Canfield WM, Gregory W and Kornfeld S. **(1992)** Characterization of the signal for rapid internalization of the bovine mannose 6-phosphate/insulin-like growth factor-II receptor. *J. Biol. Chem*. 267; 11069–11077.

- **Jirtle RL**, Carr BI and Scott CD. **(1991)** Modulation of insulin-like growth factor-II/mannose 6-phosphate receptors and transforming growth factor-beta 1 during liver regeneration. *J. Biol. Chem*. 266; 22444–22450.

- **Johnson FK** and Kornfeld S. **(1992a)** A His-Leu-Leu sequence near the carboxy terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. *J.Biol.Chem*. 267; 17110–17115.

- **Johnson KF** and Kornfeld S. **(1992b)** The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol*. 119; 249–257.

- **Kandror KV** and Pilch PF. **(1988)** Multiple endosomal recycling pathways in rat adipose cells. *Biochem. J.* 331(Pt 3); 829–835.

- **Kang JX**, Bell J, Beard RL and Chandraratna RA. **(1999)** Mannose 6-phosphate/ insulin-like growth factor II receptor mediates the growth-inhibitory effects of retinoids. *Cell Growth Differ*. 10; 591–600.

- **Kang JX**, Bell J, Leaf A, Beard RL and Chandraratna RA. **(1998)** Retinoic acid alters the intracellular trafficking of the mannose-6-phosphate/ insulin-like growth factor II receptor and lysosomal enzymes. *Proc. Natl. Acad. Sci. U.S.A*. 95; 13687–13691.

- **Kang JX**, Li Y and Leaf A. **(1997)** Mannose-6-phosphate/insulin-like growth factor-II receptor is a receptor for retinoic acid. *Proc. Natl. Acad. Sci. U.S.A*. 94; 13671–13676.

- **Kaplan A**, Achord DT and Sly WS. **(1977)** Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 74; 2026–2030.

- **Kasper D**, Dittmer F, von Figura K and Pohlmann R. **(1996)** Neither type of mannose 6-phosphate receptor is sufficient for targeting of lysosomal enzymes along intracellular routes. *J. Cell Biol*. 134; 615–623.

- **Kelly TE**, Thomas GH, Taylor HA Jr, McKusick VA, Sly WS, Glaser JH, Robinow M, Luzzatti L, Espiritu C, Feingold M, Bull MJ, Ashenhurst EM and Ives EJ. **(1975)** Mucolipidosis III (pseudo-Hurler polydystrophy): clinical and laboratory studies in a series of 12 patients. *Johns Hopkins Med. J.* 137; 156–175.

- **Kiely M**, McKnight GS and Schimke R. **(1976)** Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. *J.Biol.Chem*. 251; 5490–5495.

- **Kiess W**, Blickenstaff G D, Sklar MM, Thomas C L, Nissley SP and Sahagian GG. **(1988)** Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *J. Biol. Chem.* 263; 9339–9344.

- **Kiess W**, Greenstein LA, Lee L, Thomas C and Nissley SP. **(1991)** Biosynthesis of the insulin-like growth factor-II (IGF-II)/mannose-6-phosphate receptor in rat C6 glial cells: the role of N-linked glycosylation in binding of IGF-II to the receptor. *Mol. Endocrinol*. 5; 281–291.

- **Kiess W**, Yang Y, Kessler U and Hoeflich A. **(1994)** Insulin-like growth factor II (IGF-II) and the IGF-II/mannose-6-phosphate receptor: the myth continues. *Horm. Res*. 41; 66–73.

- **Killian JK**, Buckley TR, Stewart N, Munday BL and Jirtle RL. **(2001)** Marsupials and Eutherians reunited: genetic evidence for the Theria hypothesis of mammalian evolution. *Mamm. Genome*. 12; 513–517.

- **Killian JK**, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, MacDonald RG and Jirtle RL. **(2000)** M6P/IGF2R imprinting evolution in mammals. *Mol. Cell* 5; 707–716.

- **Killian JK** and Jirtle RL. **(1999)** Genomic structure of the human M6P/IGF2 receptor. *Mamm. Genome.* 10; 74–77.

- **Kleeberger L** and Rottinger EM. **(1993)** Effect of pH and moderate hyperthermia on doxorubicin, epirubicin and aclacinomycin A cytotoxicity for Chinese hamster ovary cells. *Cancer Chemother. Pharmacol.* 33; 144–148.

- **Klier HJ**, von Figura K and Pohlmann R. **(1991)** Isolation and analysis of the human 46-kDa mannose 6-phosphate receptor gene. *Eur. J. Biochem*. 197; 23–28.

- **Korner C**, Herzog A, Weber B, Rosorius O, Hemer F, Schmidt B and Braulke T. **(1994)** In vitro phosphorylation of the 46-kDa mannose 6-phosphate receptor by casein kinase: II. Structural requirements for efficient phosphorylation. *J. Biol. Chem*. 269; 16529–16532.

- **Korner C**, Nurnberg B, Uhde M and Braulke T. **(1995)** Mannose 6-phosphate/insulin-like growth factor II receptor fails to interact with Gproteins, analysis of mutant cytoplasmic receptor domains. *J. Biol. Chem*. 270; 287–295.

- **Kornfeld R**, Bao M, Brewer K, Noll C and Canfield WM. **(1998)** Purification and multimeric structure of bovine α-*N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase. *J Biol Chem*. 273; 23203–23210.

- **Kornfeld S**. **(1987)** Trafficking of lysosomal enzymes. *FASEB J.* 1; 462–468.

- **Kornfeld S. (1992)** Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu. Rev. Biochem*. 61; 307–330.

- **Kornfeld S** and Mellman I. **(1989)** The biogenesis of lysosomes. *Annu. Rev. Cell Biol*. 5; 483–525.

- **Kornfeld S** and Sly WS. **(1995)** I-cell disease and pseudo-Hurler polydystrophy: disorders of lysosomal enzyme phosphorylation and localization. *In*: C.R. Scriver (ed.): *The metabolic and molecular bases of inherited disease*, seventh edition, McGraw-Hill, Inc., New York, pp 2495–2508.

- **Köster A**, Nagel G, von Figura K and Pohlmann R. **(1991)** Molecular cloning of the mouse 46 kDa mannose 6-phosphate receptor (MPR 46). *Biol.Chem.Hoppe-Seyler*. 372; 297–300.

- **Köster A**, Saftig P, Matzner U, von Figura K, Peters C and Pohlmann R. **(1993)** Targeted disruption of the M(r) 46,000 mannose 6-phosphate receptor gene in mice results in misrouting of lysosomal proteins. *EMBO J.* 12(13); 5219–5223.

- **Köster A**, von Figura K and Pohlmann R. **(1994)** Mistargeting of lysosomal enzymes in M(r) 46,000 mannose 6-phosphate receptor-deficient mice is compensated by carbohydrate-specific endocytotic receptors. *Eur. J. Biochem*. 224; 685–689.

- **Kostrouch Z**, Munari-Silem Y, Rajas F, Bernier-Valentin F and Rousset B. **(1991)** Thyroglobulin internalized by thyrocytes passes through early and late endosomes. *Endocrinology* 129; 2202–2211.

- **Kyle JW**, Nolan CM, Oshima A and Sly WS. **(1988)** Expression of human cation-independent mannose 6-phosphate receptor cDNA in receptor negative mouse $P388D_1$ cells following gene transfer. *J.Biol.Chem*. 263; 16230–16235.

- **Laemmli UK**. **(1970)** Cleavage of structural proteins during the assembly of the head of T bacteriophage. *Nature* 227; 680–685.

- **Lau MM**, Stewart CE, Liu Z, Bhatt H, Rotwein P and Stewart CL. **(1994)** Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev*. 8; 2953–2963.

- **Laureys G**, Barton DE, Ullrich A and Francke U. **(1988)** Chromosomal mapping of the gene for the type II insulin-like growth factor receptor/cation-independent mannose 6-phosphate receptor in man and mouse. *Genomics* 3; 3224–3229.

- **Lazzarino DA** and Gabel CA. **(1988)** Biosynthesis of the mannose 6-phosphate recognition marker in transport-impaired mouse lymphoma cells. Demonstration of a two-step phosphorylation. *J.Biol.Chem*. 263; 10118–10126.

- **Le Borgne R** and Hoflack B. **(1998)** Protein transport from the secretory to the endocytic pathway in mammalian cells. *Biochim. Biophys. Acta*. 1404; 195–209.

- **Lee SJ** and Nathans D. **(1988)** Proliferin secreted by cultured cells binds to mannose 6-phosphate receptors. *J. Biol. Chem*. 263; 3521– 3527.

- **Lemansky P** and Herzog V. **(1992)** Endocytosis of thyroglobulin is not mediated by mannose-6-phosphate receptors in thyrocytes. Evidence for low-affinity-binding sites operating in the uptake of thyroglobulin. *Eur. J. Biochem*. 209; 111–119.

- **Leroy JG** and DeMars RI. **(1967)** Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* 157; 804–806.

- **Li M**, Distler JJ and Jourdian GW. **(1990)** The aggregation and dissociation properties of a low molecular weight mannose 6-phosphate receptor from bovine testis. *Arch. Biochem. Biophys*. 283; 150–157.

- **Lightbody J**, Weissmann U, Hadorn B and Herschkowitz N. **(1971)** I-cell disease: multiple lysosomal-enzyme defect. *Lancet.* 1; 451.

- **Linnell J**, Groeger G and Hassan AB. **(2001)** Real time kinetics of insulinlike growth factor II (IGF-II) interaction with the IGF-II/mannose 6-phosphate receptor. The effects of domain 13 and pH. *J. Biol. Chem*. 276; 23986–23991.

- **Liu Z**, Mittanck DW, Kim S and Rotwein P. **(1995)** Control of insulin-like growth factor-II/mannose 6-phosphate receptor gene transcription by proximal promoter element. *Mol. Endocrinol*. 9; 1477–1487.

- **Lobel P**, Dahms NM, Breitmeyer J, Chirgwin JM and Kornfeld S. **(1987)** Cloning of the bovine 215 kDa cation-independent mannose 6-phosphate receptor. *Proc. Natl. Acad. Sci. U.S.A*. 84; 2233–2237.

- **Lobel P**, Dahms NM and Kornfeld S. **(1988)** Cloning and sequence analysis of the cation-independent mannose 6-phosphate receptor. *J. Biol. Chem*. 263; 2563–2570.

- **Lobel P**, Fujimoto K, Ye RD, Griffiths G and Kornfeld S. **(1989)** Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. *Cell* 57; 787–796.

- **Ludwig T**, Eggenschwiler J, Fisher P, D'Ercole AJ, Efstratiadis ML and Efstratiadis A. **(1996)** Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Dev. Biol*. 177; 517–535.

- **Ludwig T**, Munier-Lehmann H, Bauer U, Hollinshead M, Ovitt C, Lobel P and Hoflack B. **(1994)** Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts. *EMBO J*. 13; 3430–3437.

- **Ludwig T**, Ovitt CE, Bauer U, Hollinshead M, Remmler J, Lobel P, Ruther U and Hoflack B. **(1993)** Targeted disruption of the mouse cation-dependent mannose 6-phosphate receptor results in partial missorting of multiple lysosomal enzymes. *EMBO J.* 12; 5225–5235.

- **Ludwig T**, Ruther U, Metzger R, Copeland NG, Jenkins NA, Lobel P and Hoflack B. **(1992)** Gene and pseudogene of the mouse cationdependent mannose 6-phosphate receptor. Genomic organization, expression, and chromosomal localization. *J. Biol. Chem*. 267; 12211–12219.

- **Ma ZM**, Grubb JH and Sly WS. **(1991)** Cloning, sequencing and functional characterization of the murine 46 kDa mannose 6-phosphate receptor. *J.Biol.Chem*. 266; 10589–10595.

- **Ma ZM**, Grubb JH and Sly WS. **(1992)** Divalent cation-dependent stimulation of ligand binding to the 46-kDa mannose 6-phosphate receptor correlates with divalent cation-dependent tetramerization. *J. Biol. Chem*. 267; 19017–19022.

- **MacDonald RG** and Czech MP. **(1985)** Biosynthesis and processing of the type II insulin-like growth factor receptor in H-35 hepatoma cells. *J. Biol. Chem*. 260; 11357–11365.

- **MacDonald RG**, Pfeffer SR, Coussens L, Tepper MA, Brocklebank CM, Mole JE, Anderson JK, Chen E, Czech MP and Ullrich A. **(1988)** A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* 239; 1134–1137.

- **Mainferme F**, Wattiaux R and von Figura K. **(1985)** Synthesis, transport and processing of cathepsin C in Morris hepatoma 7777 cells and rat hepatocytes. *Eur J Biochem.* 153 (1); 211–216.

- **Marron-Terada PG**, Bollinger KE and Dahms NM. **(1998)** Characterization of truncated and glycosylation-deficient forms of the cation-dependent mannose 6-phosphate receptor expressed in baculovirus-infected insect cells. *Biochemistry* 37; 17223–17229.

- **Marron-Terada PG**, Hancock MK, Haskins DJ and Dahms NM. **(2000)** Recognition of *Dictyostelium discoideum* lysosomal enzymes is conferred by the amino-terminal carbohydrate binding site of the insulinlike growth factor II/mannose 6-phosphate receptor. *Biochemistry* 39; 2243–2253.

- **Matzner U**, Hille-Rehfeld A, von Figura K and Pohlmann R. **(1996)** Expression of mannose 6-phosphate receptors in chicken. *Dev. Dyn*. 207; 11–24.

- **Matzner U**, von Figura K and Pohlmann R. **(1992)** Expression of the two mannose 6-phosphate receptors is spatially and temporally different during mouse embryogenesis. *Development* 114; 965– 972.

- **Mendez E**, Planas JV, Castillo J, Navarro I and Gutierrez J. **(2001)** Identification of a type II insulin-like growth factor receptor in fish embryos. *Endocrinology* 142; 1090–1097.

- **Meyer C**, Eskelinen EL, Guruprasad MR, von Figura K and Schu P. **(2001)** Mu1A deficiency induces a profound increase in MPR300/IGF-II receptor internalization rate. *J. Cell Sci*. 114; 4469–4476.

- **Meyer C**, Zizioli D, Lausmann S, Eskelinen EL, Hamann J, Saftig P, von Figura K and Schu P. **(2000)** Mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. *EMBO J*. 19; 2193–2203.

- **Morgan DO**, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA and Rutter WJ. **(1987)** Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* 329; 301–307.

- **Motyka B**, Korbutt G, Pinkoski MJ, Heibein JA, Caputo A, Hobman M, Barry M, Shostak I, Sawchuk T, Holmes CF, Gauldie J and Bleackley RC. **(2000)** Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cellinduced apoptosis. *Cell* 103; 491–500.

- **Mullins C** and Bonifacino JS. **(2001)** The molecular machinery for lysosome biogenesis. *BioEssays* 23; 333–343.

- **Mullock BM**, Perez JH, Kuwana T, Gray SR, and Luzio JP. **(1994) Lysosomes can fuse with a late endosomal compartment in a cell-free system from rat liver** *J. Cell Biol.*126; 1173–1182.

- **Munger JS**, Harpel JG, Gleizes PE, Mazzieri R, Nunes I and Rifkin DB. **(1997)** Latent transforming growth factor-beta—structural features and mechanisms of activation. *Kidney Int*. 51; 1376–1382.

- **Murray GJ** and Neville DM Jr. **(1980)** Mannose 6-phosphate receptor-mediated uptake of modified low density lipoprotein results in down regulation of hydroxymethylglutaryl-CoA reductase in normal and familial hypercholesterolemic fibroblasts. *J. Biol. Chem*. 255; 11942–11948.
- **Nakagawa T**, Setou M, Seog D, Ogasawara K, Dohmae N, Takio K and Hirokawa N. **(2000)** A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex. *Cell* 103; 569–581.

- **Natowicz MR**, Chi MM-Y, Lowry OH and Sly WS. **(1979)** Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor-mediated pinocytosis of b-glucuronidase by human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A*. 76; 4322–4326.

- **Nishikawa A**, Gregory W, Frenz J, Cacia J and Kornfeld S. **(1997)** The phosphorylation of bovine DNase I Asn-linked oligosaccharides is dependent on specific lysine and arginine residues. *J. Biol. Chem*. 272; 19408–19412.

- **Nishikawa A**, Nanda A, Gregory W, Frenz J and Kornfeld S. **(1999)** Identification of amino acids that modulate mannose phosphorylation of mouse DNase I, a secretory glycoprotein. *J. Biol. Chem*. 274; 19309–19315.

- **Nissley P**, Kiess W and Sklar M. **(1993)** Developmental expression of the IGF-II/mannose 6-phosphate receptor. *Mol. Reprod. Dev*. 35; 408–413.

- **Nissley P** and Kiess W. **(1991)** Reciprocal modulation of binding of lysosomal enzymes and insulin-like growth factor-II (IGF-II) to the mannose 6-phosphate/IGF-II receptor. *Adv. Exp. Med. Biol*. 293; 311–324.

- **Nolan CM**, Kyle JW, Watanabe H and Sly WS. **(1990)** Binding of insulin-like growth factor II (IGF-II) by human cation-independent mannose 6-phosphate receptor/IGF-II receptor expressed in receptor-deficient mouse L cells. *Cell Regul*. 1(2); 197–213.

- **Nunes I**, Shapiro RL and Rifkin DB. **(1995)** Characterization of latent TGFbeta activation by murine peritoneal macrophages. *J. Immunol*. 155; 1450–1459.

- **Nykjaer A**, Christensen EI, Vorum H, Hager H, Petersen CM, Roigaard H, Min HY, Vilhardt F, Moller LB, Kornfeld S and Gliemann J. **(1998)** Mannose 6-phosphate/insulin-like growth factor-II receptor targets the urokinase receptor to lysosomes via a novel binding interaction. *J. Cell Biol*. 141; 815–828.

- **Oates AJ**, Schumaker LM, Jenkins SB, Pearce AA, DaCosta SA , Arun B and Ellis MJ. **(1998)** The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), a putative breast tumour suppressor gene. *Breast Cancer Res. Treat*. 47; 269–281.

- **O'Dell SD** and Day IN. **(1998)** Insulin-like growth factor II (IGF-II). *Int. J. Biochem. Cell Biol*. 30; 767–771.

- **Ohnuma K**, Munakata Y, Ishii T, Iwata S, Kobayashi S, Hosono O, Kawasaki H, Dang NH and Morimoto C. **(2001)** Soluble CD26/dipeptidyl peptidase IV induces T cell proliferation through CD86 upregulation on APCs. *J. Immunol*. 167; 6745–6755.

- **Oka Y**, Rozek LM and Czech MP. **(1985)** Direct demonstration of rapid insulin-like growth factor II receptor internalization and recycling in rat adipocytes. Insulin stimulates 125I-insulin-like growth factor II degradation by modulating the IGF-II receptor recycling process. *J. Biol. Chem*. 260; 9435–9442.

- **Olivia C. Probst**, Phuong Ton, Barbara Svoboda, Andrew Gannon, Werner Schuhmann, Johannes Wieser, Regina Pohlmann and Lukas Mach. **(2006)** The 46-kDa mannose 6-phosphate receptor does not depend on endosomal acidification for delivery of hydrolases to lysosomes. *J. Cell Science* 119; 4935–4943.

- **Orsel JG**, Sincock PM, Krise JP and Pfeffer SR. **(2000)** Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein. *Proc. Natl. Acad. Sci. U.S.A*. 97; 9047–9051.

- **Olson TS** and Lane MD. **(1989)** A common mechanism for posttranslational activation of plasma membrane receptors? *FASEB J*. 3; 1618–1624.

- **Olson LJ**, Zhang J, Dahms NM and Kim JJP. **(2002)** Twists and turns of the cation-dependent mannose 6-phosphate receptor: ligand-bound versus ligand-free receptor. *J. Biol. Chem*. 277; 10156–10161.

- **Olson LJ**, Zhang J, Lee YC, Dahms NM and Kim JJP. **(1999)** Structural basis for recognition of phosphorylated high mannose oligosaccharides by the cation-dependent mannose 6-phosphate receptor. *J. Biol. Chem*. 274; 29889–29896.

- **Oshima A**, Nolan CM, Kyle J, Grubb HJ and Sly WS. **(1988)** The human cation-independent mannose 6-phosphate receptor. Cloning and sequence of the full-length cDNA and expression of the functional receptor in COS-cells. *J. Biol. Chem*. 263; 2553–2562.

- **Pearse BM**. **(1988)** Receptors compete for adaptors found in plasma membrane coated pits. *EMBO J*. 7; 3331–3336.

- **Pohlmann R**, Boeker MW and von Figura K. **(1995)** The two mannose 6-phosphate receptors transport distinct complements of lysosomal proteins. *J. Biol. Chem*. 270; 27311–27318.

- **Pohlmann R**, Nagel G, Schmidt B, Stein M, Lorkowski G, Krentler C, Cully J, Meyer HE, Grzeschik KH, Mersmann G, Hasilik A and von Figura K. **(1987)** Cloning of a cDNA encoding the human cation-dependent mannose 6-phosphate-specific receptor. *Proc. Natl. Acad. Sci. U.S.A*. 84; 5575–5579.

- **Press B**, Feng Y, Hoflack B and Wandinger-Ness A. **(1998)** Mutant Rab7 causes the accumulation of cathepsin D and cation-independent mannose 6-phosphate receptor in an early endocytic compartment. *J. Cell Biol*. 140; 1075–1089.

- **Praveen Kumar A** and Siva Kumar N. **(2008)** Molecular cloning, expression and functional characterization of the chicken cation dependent mannose 6-phosphate receptor protein. *Int. J. Biol. Macromol*. 43(3); 300–306

- **Praveen Kumar A**, Suresh K and Siva Kumar N. **(2007)** Biochemical and Functional Characterization of cation dependent (Mr 46,000) Goat Mannose 6-phosphate Receptor. *Gycoconjugate J*. 24; 221–229

- **Puertollano R**, Aguilar RC, Gorshkova I, Crouch RJ and Bonifacino JS. **(2001)** Sorting of mannose 6-phosphate receptors mediated by the GGAs. *Science* 292; 1712–1716.

- **Punnonen EL**, Wilke T, von Figura K and Hille-Rehfeld A. **(1996)** The oligomeric state of 46-kDa mannose 6-phosphate receptor does not change upon intracellular recycling and binding of ligands. *Eur. J. Biochem*. 237; 809–818.

- **Purchio AF**, Cooper JA, Brunner AM, Lioubin MN, Gentry LE, Kovacina KS, Roth RA and Marquardt H. **(1988)** Identification of mannose 6-phosphate in two asparagine-linked sugar chains of recombinant transforming growth factor-beta 1 precursor. *J. Biol. Chem*. 263; 14211–14215.

- **Raju VSN**, von Figura K and Siva Kumar N. **(2001)** Mannose 6-phosphate receptor (MPR 300) like polypeptides in the (Arthropods and Annelidae). *Glycoconjugate. J*. 18; 292–210.

- **Roberts DL**, Weix DJ, Dahms NM and Kim JJP. **(1998)** Molecular basis of lysosomal enzyme recognition: three-dimensional structure of the cation-dependent mannose 6-phosphate receptor. *Cell* 93; 639–648.

- **Robert J. Wood** and Mark D. Hulett. **(2008)** Cell Surface-expressed Cation-independent Mannose 6-Phosphate Receptor (CD222) Binds Enzymatically Active Heparanase Independently of Mannose 6-Phosphate to Promote Extracellular Matrix Degradation. *J. Biol. Chem*. 283(7); 4165–4176.

- **Roff CF**, Wozniak RW, Blenis J and Wang JL. **(1983)** The effect of mannose 6-phosphate on the turnover of cell surface glycosaminoglycans. *Exp. Cell Res*. 144; 333–344.

- **Rohrer J** and Kornfeld R. **(2001)** Lysosomal hydrolase mannose 6-phosphate uncovering enzyme resides in the trans-Golgi network. *Mol. Biol. Cell* 12; 1623–1631.

- **Rothman JE**, Katz FN and Lodish HF. **(1978)** Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. *Cell* 15; 1447–1454.

- **Sahagian GG. (1984)** The mannose 6-phosphate receptor: function, biosynthesis and translocation. *Biol. Cell* 51; 207–214.

- **Sahagian GG** and Neufeld EF. **(1983)** Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured chinese hamster ovary cells. *J. Biol. Chem*. 258; 7121–7128.

- **Saris JJ**, Derkx FH, De Bruin RJ, Dekkers DH, Lamers JM, Saxena PR, Schalekamp MA and Jan Danser AH. **(2001a)** High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to rennin. *Am. J. Physiol., Heart Circ. Physiol*. 280; H1706–H1715.

- **Saris JJ**, Derkx FH, Lamers JM, Saxena PR, Schalekamp MA and Danser AH. **(2001b)** Cardiomyocytes bind and activate native human prorenin: role of soluble mannose 6-phosphate receptors. *Hypertension* 37; 710–715.

- **Scheel G** and Herzog V. **(1989)** Mannose 6-phosphate receptor in porcine thyroid follicle cells. Localization and possible implications for the intracellular transport of thyroglobulin. *Eur. J. Cell Biol*. 49; 140–148.

- **Schmidt B**, Kiecke-Siemsen C, Waheed A, Braulke T and von Figura K. **(1995)** Localization of the insulin-like growth factor II binding site to amino acids 1508–1566 in repeat 11 of the mannose 6-phosphate/insulin-like growth factor II receptor. *J. Biol. Chem*. 270; 14975–14982.

- **Schweizer A**, Kornfeld S and Rohrer J. **(1996)** Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. *J. Cell Biol*. 132; 577–584.

- **Schweizer A**, Kornfeld S and Rohrer J. **(1997)** Proper sorting of the cationdependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail. Proc. Natl. Acad. Sci. U.S.A. 94; 14471–14476.

- **Scott CD** and Weiss J. **(2000)** Soluble insulin-like growth factor II/mannose 6-phosphate receptor inhibits DNA synthesis in insulin-like growth factor II sensitive cells. *J. Cell Physiol*. 182; 62–68.

- **Siva Kumar N**, International Glycoconjugate Meeting GLYCO-19 Meeting, Glycocojugate J. 24, abstract No. 415, July 15-20th, **2007**, Cairns, Australia. Page 382.

- **Siva Kumar N**, Bernhard S, von Figura K and Hille A. **(1991)** Antibodies against the cytoplasmic tail can differentiate between the quaternary forms of the Mr 46000 mannose 6-phosphate receptor. *FEBS Lett* 280; 61–64.

- **Siva Kumar N**, Hille-Rehfeld A and von Figura K. **(1997)** Mannose 6-phosphate receptor proteins from reptiles and amphibians: evidence for the presence of MPR 300 and MPR 46. *Comp. Biochem. Physiol*. 118B; 805–809.

- **Siva Kumar N**, Nirmala P and Suresh K. **(2004)** Biochemical and immunological characterization of a glycosylated α-fucosidase from the invertebrate *Unio.* Interaction of the enzyme with its in vivo binding partners. *Prot.Exp.and Pur*. 37; 279–287.

- **Siva Kumar N**, Raju VSN, Suresh K, Venkatesh B, von Figura K and Pohlmann R. **(2005)** Presented at ASCB conference Sanfransisco, California, U.S.A.

- **Siva Kumar N**, Udaya Lakshmi Y, Hille-Rehfeld A and von Figura K. **(1999)** Mannose 6-phosphate receptors (MPR 300 and MPR 46) from a teleost fish (trout). *Comp. Biochem. Physiol*. 123B; 261–265.

- **Siva Kumar N** and von Figura K. **(2002)** Identification of the putative mannose 6-phosphate receptor (MPR 46) protein from the invertebrate Mollusc. *Biosci. Rep*. 22; 513–521

- **Siva Ramakrishna Y** and Siva Kumar N. **(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 J.* Available online: DOI 10.1007/s10719-008-9153-5.

- **Stein M**, Meyer HE, Hasilik A and von Figura K. **(1987a)** 46-kDa mannose 6-phosphate-specific receptor: purification, subunit composition, chemical modification. *Biol. Chem. Hoppe-Seyler* 368; 927–936.

- **Stein M**, Zijderhand-Bleekemolen JE, Geuze H, Hasilik A and von Figura K **(1987b)** Mr 46000 mannose 6-phosphate specific receptor: Its role in targeting of lysosomal enzymes. *EMBO J*. 6; 2677–2681.

- **Storch S** and Braulke T. **(2001)** Multiple C-terminal motifs of the 46-kDa mannose 6-phosphate receptor tail contribute to efficient binding of medium chains of AP-2 and AP-3. *J. Biol. Chem*. 276; 4298–4303.

- **Sudhof TC**, Goldstein JL, Brown MS and Russell DW. **(1985)** The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* 228; 815–822.

- **Suresh K**, Raju VSN, Siva Kumar N, von Figura K, Pohlmann R and Dennes A. **(2005)** The early vertebrate *Danio rerio* Mr 46000 mannose 6-phosphate receptor: biochemical and functional characterization. *Dev. Genes Evol*. 216(3); 133–143.

- **Suresh K**, Raju VSN and Siva Kumar N. **(2004)** Molecular cloning of goat Mannose 6-phophate receptors (MPR 300 and 46). *Glycoconjugate J.* 20; 257–265.

- **Suresh K**, Ramanadham M and Siva Kumar N. **(2003)** An ELISA method to quantify mannose 6-phosphate receptors. *J Biochem Biophys Meth.* 57; 237–245.

- **Suresh K**, Siva Ramakrishna Y and Siva Kumar N. **(2006)** Mannose 6-phosphate receptor (MPR 300) from Goat and chicken bind Human IGF-II. *Biosci. Rep.* 26; 101–112.

- **Szebenyi G** and Rotwein P. **(1994)** The mouse insulin-like growth factor II/cation-independent mannose 6-phosphate (IGF-II/MPR) receptor gene: molecular cloning and genomic organization. *Genomics* 19; 120–129.

- **Takatsu H**, Katoh Y, Shiba Y and Nakayama K. **(2001)** Golgi-localizing, gamma-adaptin ear homology domain, ADP-ribosylation factorbinding (GGA) proteins interact with acidic dileucine sequences within the cytoplasmic domains of sorting receptors through their Vps27p/Hrs/STAM (VHS) domains. *J. Biol. Chem*. 276; 28541–28545.

- **Tarentino AL**, Gomez CM and Plummer THJ. **(1985)** Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* 24; 4665–4671.

- **Tikkanen R**, Obermuller S, Denzer K, Pungitore R, Geuze HJ, von Figura K and Honing S. **(2000)** The dileucine motif within the tail of the MPR46 is required for sorting of the receptor in endosomes. *Traffic* 1; 631–640.

- **Todderud G** and Carpenter G. **(1988)** Presence of mannose phosphate on the epidermal growth factor receptor in A-431 cells. *J. Biol. Chem*. 263; 17893–17896.

- **Tondeur M**, Vamos-Hurwitz E, Mockel-Pohl S, Dereume JP, Cremer N and Loeb H. **(1971)** Clinical, biochemical, and ultrastructural studies in a case of chondrodystrophy presenting the I-cell phenotype in culture. *J. Pediatr.* 79; 366–378.

- **Tong PY**, Gregory W and Kornfeld S. **(1989)** Ligand interactions of the cation-independent mannose 6-phosphate receptor. The stoichiometry of mannose 6-phosphate binding. *J. Biol. Chem*. 264; 7962–7969.

- **Tong PY** and Kornfeld S. **(1989)** Ligand interactions of the cation-dependent mannose 6-phosphate receptor. *J. Biol. Chem*. 264; 7970–7975.

- **Tong PY**, Tollefsen SE and Kornfeld S. **(1988)** The cation-independent mannose 6-phosphate receptor binds insulin-like growth factor II. *J. Biol. Chem*. 263; 2585–2588.

- **Topp KS**, Rothman AL and Lavail JH. **(1997)** Herpes virus infection of RPE and MDCK cells: polarity of infection. *Exp. Eye Res*. 64; 343–354.

- **Towbin H,** Staehelin T and Gordon J. **(1979)** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A*. 76; 4350–4354.

- **Trimble RB**, Tarentino AL, Plummer THJ and Maley F. **(1978)** Asparaginyl glycopeptides with a low mannose content are hydrolyzed by endo-beta-N-acetylglucosaminidase H. *J. Biol. Chem.* 253; 4508–4511.

- **van den Eijnden MM**, Saris JJ, de Bruin RJ, de Wit E, Sluiter W, Reudelhuber TL, Schalekamp MA, Derkx FH and Danser AH. **(2001)** Prorenin accumulation and activation in human endothelial cells: importance of mannose 6-phosphate receptors. *Arterioscler., Thromb., Vasc. Biol*. 21; 911–916.

- **Udaya lakshmi Y**, Siva Kumar N, Schu P, von Figura K and Hille-Rehfeld A. **(2000)** Conserved cassette structure of vertebrate Mr 300 kDa mannose 6-phosphate receptors: partial cDNA sequence of fish MPR 300. *Comp. Biochem. Physiol*. 127; 433–441.

- **Udaya lakshmi Y**, Yalamarthy R, Hille-Rehfeld A, von Figura K and Siva Kumar N. **(1999)** Identification of the putative mannose 6-phoshate receptor protein (MPR 300) in the invertebrate *unio*. *Biosci. Rep*.15; 403–409.

- **Ullrich K**, Basner R, Gieselmann V and von Figura K. **(1979)** Recognition of Human Urine a-N-Acetylglucosaminidase by Rat Hepatocytes involvement of receptors specific for galactose, mannose 6-phosphate and mannose. *Biochem. J.* 180; 413–419

- **Ullrich K**, Mersmann G, Weber E and von Figura K. **(1978)** Evidence for Lysosomal Enzyme Recognition by Human Fibroblasts via a Phosphorylated Carbohydrate Moiety. *Biochem. J*. 170; 643–650.

- **van Kesteren CA**, Danser AH, Derkx FH, Dekkers DH, Lamers JM, Saxena PR and Schalekamp MA. **(1997)** Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cardiac cells. *Hypertension* 30; 1389–1396.

- **Volpert O**, Jackson D, Bouck N and Linzer DI. **(1996)** The insulin-like growth factor II/mannose 6-phosphate receptor is required for proliferin-induced angiogenesis. *Endocrinology* 137; 3871–3876.

- **von figura K**, Gieselmann V and Hasilik A. **(1984)** Antibody to mannose 6-phosphate specific receptor induces receptor deficiency in human fibroblasts. *EMBO J.* 3; 1281–1286

- **von Figura K** and Hasilik A. **(1986)** Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55; 167–193.

- **von Figura K** and Klein D. **(1979)** Isolation and Characterization of Phosphorylated Oligosacchardes from a-Acetyl-glucosaminidase that are Recognized by Cell-Surface Receptors. *Euro. J. of Biochem*. 94; 347–354.

- **Waheed A**, Hasilik A and von Figura K. **(1982)** Synthesis and processing of arylsulfatase A in human skin fibroblasts. *Hoppe Seylers Z Physiol Chem.* 363(4); 425–430.

- **Waheed A**, Hille A, Junghans U and von Figura K. **(1990)** Quaternary structure of the Mr 46,000 mannose 6-phosphate specific receptor: effect of ligand, pH, and receptor concentration on the equilibrium between dimeric and tetrameric receptor forms. *Biochemistry* 29; 2449–2455.

- **Waheed A** and von Figura K. **(1990)** Rapid equilibrium between monomeric, dimeric and tetrameric forms of the 46-kDa mannose 6-phosphate receptor at 37 degrees C. Possible relation to the function of the receptor. *Eur. J. Biochem*. 193; 47–54.

- **Wang ZQ**, Fung MR, Barlow DP and Wagner EF. **(1994)** Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* 372; 464–467.

- **Wang ZH**, Gershon MD, Lungu O, Zhu Z and Gershon AA. **(2000)** Trafficking of varicella-zoster virus glycoprotein gI: T(338)-dependent retention in the trans-Golgi network, secretion, and mannose 6-phosphate-inhibitable uptake of the ectodomain. *J. Virol*. 74; 6600–6613.

- **Wan L**, Molloy SS, Thomas L, Liu G, Xiang Y, Rybak SL and Thomas G. **(1998)** PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. *Cell* 94; 205–216.

- **Watanabe H**, Grubb JH and Sly WS. **(1990)** The Overexpressed Human 46-kDa Mannose 6-Phosphate Receptor Mediates Endocytosis and Sorting of β-Glucuronidase. *Proc Natl Acad Sci U.S.A.* 87; 8036–8040

- **Wendland M**, Hille A, Nagel G, Waheed A, von Figura K and Pohlmann R. **(1989)** Synthesis of a truncated Mr 46,000 mannose 6-phosphate receptor that is secreted and retains ligand binding. *Biochem. J*. 260; 201–206.

- **Wendland M**, von Figura K and Pohlmann R. **(1991)** Mutational analysis of disulfide bridges in the Mr 46,000 mannose 6-phosphate receptor. Localization and role for ligand binding. *J. Biol. Chem*. 266; 7132–7136.

- **Wendland M**, Waheed A, Schmidt B, Hille A, G. Nagel, von Figura K and Pohlmann R. **(1991)** Glycosylation of the Mr 46,000 mannose 6-phosphate receptor. Effect on ligand binding, stability, and conformation. *J. Biol. Chem*. 266; 4598–4604.

- **Wenk J**, Hille A and von Figura K. **(1991)** Quantitation of Mr 46000 and Mr 300000 mannose 6-phosphate receptors in human cells and tissues. *Biochem. Int*. 23; 723–731.

- **Westcott KR** and Rome LH. **(1988)** Cation-independent mannose 6-phosphate receptor contains covalently bound fatty acid. *J. Cell. Biochem*. 38; 23–33.

- **Westlund B**, Dahms NM and Kornfeld S. **(1991)** The bovine mannose 6-phosphate/insulin-like growth factor II receptor. Localization of mannose 6-phosphate binding sites to domains 1– 3 and 7 – 11 of the extracytoplasmic region. *J. Biol. Chem*. 266; 23233–23239.

- **Willingham MC**, Pastan IH and Sahagian GG. **(1983)** Ultrastructural immunocytochemical localization of the phosphomannosyl receptor in Chinese hamster ovary (CHO) cells. *J. Histochem. Cytochem*. 31; 1–11.

- **Wraith J. E**. **(2002)** Lysosomal disorders. *Semin Neonatol*; 7; 75–83.

- **Yandell CA**, Dunbar AJ, Wheldrake JF and Upton Z. **(1999)** The kangaroo cation-independent mannose 6-phosphate receptor binds insulinlike growth factor II with low affinity. *J. Biol. Chem*. 274; 27076–27082.

- **Yang YW**, Robbins AR, Nissley SP and Rechler MM. **(1991)** The chick embryo fibroblast cation-independent mannose 6-phosphate receptor is functional and immunologically related to the mammalian insulinlike growth factor-II (IGF-II)/man 6-P receptor but does not bind IGF-II. *Endocrinology* 128; 1177–1189.

- **York SJ**, Arneson LS, Gregory WT, Dahms NM and Kornfeld S. **(1999)** The rate of internalization of the mannose 6-phosphate/insulin-like growth factor II receptor is enhanced by multivalent ligand binding. *J. Biol. Chem*. 274; 1164–1171.

- **Zhang Q**, Berggren PO and Tally M. **(1997)** Glucose increases both the plasma membrane number and phosphorylation of insulin-like growth factor II/mannose 6-phosphate receptors. *J. Biol. Chem*. 272; 23703–23706.

- **Zhang Y** and Dahms NM. **(1993)** Site-directed removal of N-glycosylation sites in the bovine cation-dependent mannose 6-phosphate receptor: effects on ligand binding, intracellular targetting and association with binding immunoglobulin protein. *Biochem. J*. 295; 841–848.

- **Zhou**, Zhongmin MA and Sly WS. **(1995)** Cloning and expression of the cDNA of chicken cation-independent mannose-6-phosphate receptor. *Proc. Natl. Acad. Sci. U.S.A*. 92; 9762–9766.

- **Zhu Y**, Doray B, Poussu A, Lehto VP and Kornfeld S. **(2001)** Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor. *Science* 292; 1716–1718.

- **Zhu Z**, Gershon MD, Ambron R, Gabel C and Gershon AA. **(1995)** Infection of cells by varicella zoster virus: inhibition of viral entry by mannose 6-phosphate and heparin. *Proc. Natl. Acad. Sci. U.S.A*. 92; 3546–3550.