Mannose 6-phosphate receptors (MPR 300 and 46) from goat and chicken :: Molecular cloning of goat receptors

Thesis submitted for the Degree of
DOCTOR OF **PHILOSOPHY**

By

KODURU SURESH

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

Enrolment No: **2KLBPH10** September 2003

University of Hyderabad
School of Life Sciences,
Department of Biochemistry
Hyderabad 500046 -INDIA

CERTIFICATE

This is to certify that this thesis entitled **"Mannose 6-phosphate receptors (MPR 300 and 46) from goat and chicken :: Molecular cloning of goat receptors"** submitted to the University of Hyderabad by **Mr. KODURU SURESH** 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.

Dr. N. Siva Kumar 15/09/03
Supervisor

Head 15/9/03
Department of Biochemistry

Dean, 15/9/03
School of Life Sciences

University of Hyderabad
School of Life Sciences,
Department of Biochemistry
Hyderabad 500046 -INDIA

DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Dr. N. Siva Kumar. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

K. Suresh 15/9/03

K.Suresh

Date: 15-09-03

Dr.N.Siva Kumar
(Supervisor)

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr.N.Siva ***Kumar,*** *for his excellent guidance and support given during the course of this study. His ideas and views have paved way to complete my work successfully. I will always be indebted to him for his generosity.*

I am thankful to Prof. T.Suryanarayana, Head, Department of Biochemistry and Dean, School of Life Sciences for providing necessary facilities to carry out my work.

I am also thankful to Prof. K. Subba Rao, former Head, Department of Biochemistry, Prof. M. Ramanadham, Prof.K. VA.Ramaiah, (for useful discussions, suggestions and for allowing me to use most of their lab facilities), other faculty members of the Department of Biochemistry and School of Life Sciences for their timely help and special thanks to Dr.K. Anand Kumar for permitting me to use the PCR machine in his lab.

I wish to thank Dr.M.J.Swamy (School of Chemistry, University of Hyderabad) and his group (especially Dr. Ramakrishnan, Miss.Roopa and MrAnbu) for helpful discussions.

I am very thankful to Prof. P.B.Kirthi, for allowing me to use necessary equipment for Northern blot analysis.

I am very thankful to Prof.Dr.Dr.h.c. Kurt von Figura, Goettingen University, Germany, for providing synthetic peptides and MSC1 antibody and for useful suggestions.

I wish to thank Prof. Dr. T. Braulke, University of Hamburg, Germany, for providing Biotinylated IGF-II.

I also express my sincere thanks to the family members of Dr.N.Siva Kumar for their encouragement and moral support.

I would like to say thanks to all my lab members, Dr. Udaya Lakshmi, Dr. Radha, VSN Raju, Lavanya, Siva Rama Krishna, Nagender and Venkataramana for their help.

/ am happy to acknowledge the valuable and timely help rendered by Hafiz, Chenna Reddy, Reddaiah, Madhan Mohan, Nagabhushan, Rajasekhar, Hari Krishna, Ram Prasad, Mallikarjun Reddy, Hussain, Mahipal, Markandeya, Chendra Sekahr, Neelima, Padmaja, Dheeraj, Roda, Kiran, Ravindra, Vijaya Prasad (Major), Gargi, Kalavathi, Nageswari, Sreeni, Zeenath, Subhashini, Aparna, Sudar, Sripriya, research scholars, School of Life Sciences.. I also thank Bala Krishna Reddy, Gupta, Mr.Vartul and Sailu (CDFD).

My special thanks to Krishna Murthy, Prem Syamsundar, Harish, Kiran and Sampth.

I am happy to thank Nirmala, Sreehari, Praveen, Madhu Babu and Eswar Reddy for their valuable and timely help.

I wish to thank Mr.Gopala Krishna (Guru Swamy) and family.

My sincere thanks to the non-teaching staff of the Department of Biochemistry, University of Hyderabad.

I am extremely grateful to my hardworking parents, whose endeavors made me what I am. I am also thankful to my brother and uncle for their love, affection and encouragement throughout my academic life.

And I am also thankful to the Department of Biochemistry and University of Hyderabad for giving this opportunity to pursue my doctoral degree.

I greatly acknowledge the financial support received form DST,(SP/SO/D21197) New Delhi.

-Koduru Suresh

CONTENTS

5. **CHAPTER V**

Molecular cloning of goat receptors

Abbreviations

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

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

List of Figures

List of Tables

CHAPTER -I

INTRODUCTION

Lysosomes are acidic cytoplasmic vacuoles, that contain many hydrolytic enzymes, synthesized in the rough endoplasmic reticulum which are targeted to their final destination by mannose 6-phosphate receptors (for reviews see von Figura and Hasilik, 1986; Pfeffer, 1988; Kornfeld and Mellamn, 1989; Kornfeld, 1992; Hille-Rehfeld, 1995; Pohlmann, 1996; Dahms and Hancock, 2002). These hydrolases are responsible for the degradation of both internalized and endogeneous (endocytic and autophagic pathway) macromolecules into simpler substances (Kornfeld and Mellamn, 1989; Mullins and Bonifacino, 2001). Some examples of lysosomal enzymes are proteases, glycosidases, nucleases, phosphotases, Upases etc. In mammals, the site at which these enzymes are synthesized and the mechanism by which these are ferried to lysosomes is well established.

Lysosomal enzyme sorting and transport involves the following steps:

- Lysosomal enzymes (acid hydrolases) are synthesized on membrane bound ribosomes along with other proteins on the rough endoplasmic reticulum (RER). They then penetrate into the lumen of the rough endoplasmic reticulum after which the amino terminal hydrophobic signal sequence is cleaved (Walter and Blobel, 1982; Gilmore *et al.*, 1982; Meyer *et al,* 1982). These proteins are co-translationally glycosylated by the transfer of high mannose oligosaccharides to specific asparagine residues (Kiely *et al,* 1976; Bergman *et al,* 1978; Rothman *et al,* 1978). Usually this transfer takes place prior to folding of the protein. As these newly synthesized glycoproteins migrate through the endoplasmic reticulum (ER) to the Golgi apparatus, oligosaccharide chains are further processed in a differential manner (Tabas and Kornfeld, 1980).

- **Biosynthesis of recognition marker in lysosomal enzymes:** This occurs in the *cis* region of the Golgi apparatus (Pohlmann *et al.*, 1982; Pelham, 1988), where the newly synthesized acid hydrolases are recognized and acted upon by two distinct enzymes, thought to be localized in the pre-Golgi compartment and the *cis*-Golgi compartment respectively (Lazzarino and Gabel, 1988, 1989). The first enzyme N-acetylglucosaminyl 1-phosphotransferase, transfers N-acetylglucosaminyl 1-phosphate from UDP-N-acetyl glucosamine to the 6-hydroxyl group of a terminal mannose residue on the high mannose oligosaccharide chains of the acid hydrolases (Reitman and Kornfeld, 1981). The second enzyme, N-acetylglucosaminidase, removes the N-acetyl glucosamine residue which is in phosphodiester linkage to the mannose residue on the oligosaccharide chains thus resulting in the generation of high affinity mannose 6-phosphate monoesters (Varki and Kornfeld, 1980; Waheed *et al.*, 1981). Thus, hydrolases selectively acquire high affinity mannose 6-phosphate, which serves as recognition marker for mannose 6-phosphate receptors (MPRs) (Figure 1) (Sly and Fisher, 1982; von Figura and Hasilik, 1986).

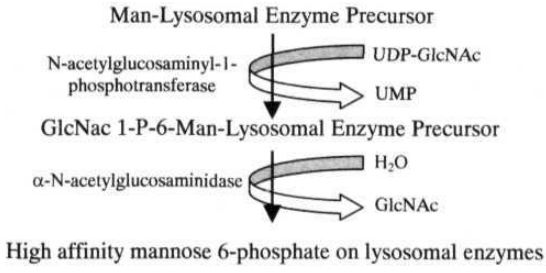


Figure 1: Schematic representation of the two step biosynthetic process for the generation of terminal mannose 6-phosphate recognition marker in lysosomal enzymes.

In humans, absence of this recognition system leads to severe lysosomal storage disease emphasizing an essential role in the biogenesis of lysosomes (von Figura and Hasilik, 1986). This could be seen particularly in I-cell disease, where lysosomal enzymes fail to acquire the mannose 6-phosphate residue (Hasilik and Neufeld 1980; Bach *et al.*, 1979), leading to the accumulation of undigested substrates (inclusions) in the cells. Table 1 shows some of the lysosomal enzymes and their disorders.

ENZYME DEFICIENCY	DISORDER
p-hexosaminidase	Tay-Sachs disease
p-hexosaminidase	Sandhoff disease (A severe form of Tay-Sachs disease)
β-galactosidase	GM1 -gangliosidosis
Glucocerebrosidase	Gaucher's disease
Galactocerebrosidase	Krabbe disease
Arylsulphatase A	Metachromatic leucodystrophy
α-galactosidase A	Fabry's disease
Ceramidase	Farber disease
Spingomyelinase	Niemann-Pick disease types A & B

Table 1. Few lysosomal enzymes and their disorders.

- This level of recognition involves segregation and binding of these high affinity ligands by specific membrane receptors designated as mannose 6-phosphate receptors (MPRs) in the *trans-Go\gi* apparatus. The MPRs residing within the *trans*-Golgi and plasma membrane are integral membrane glycoproteins whose expression levels have been found to vary in different tissues of humans, goat and chicken (Wenk *et al,* 1991; Suresh *et al,* 2002). The receptors after binding to the acid hydrolases are concentrated and pinched off into clathrin coated vesicles (Brown and Farquhar 1984).

The major coat proteins of these carrier vesicles are clathrin and assembly proteins (APs) also referred to as adapter proteins (Morris *et al,* 1989; Pearse *et al,* 1990; Robinson, 1992). Two types of heterotetrameric AP complexes have been identified. Localization studies indicate that AP-1 is specific for Golgi-derived vesicles, whereas AP-2 is specific for plasma membrane derived vesicles. Although it remains unclear how APs can specifically interact with a given membrane, *in vitro* studies indicate that APs have two main functions. First, they promote the polymerization of clathrin triskelions onto the membranes (Morris *et al,* 1989; Schmidt, 1992; Anderson, 1993). Second, APs appear to recognize specific protein motifs in the cytoplasmic domain of *trans*-membrane proteins, thereby mediating their recruitment into carrier vesicles (Pearse *et al,* 1990). These vesicles then fuse with the prelysosomal compartment where the receptor-ligand complexes dissociates by virtue of low pH (Figure 2).

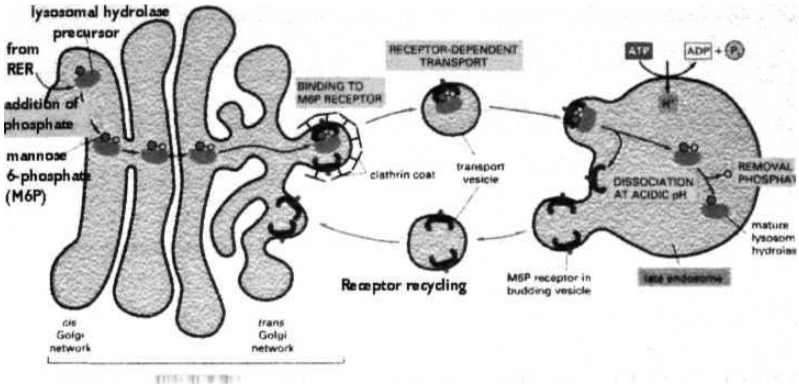


Figure 2: *Model of mannose 6-phosphate receptor mediated lysosomal enzyme targeting. (http://cellhio. utmb. edu/cellbio/lysosome. htm). After synthesis and acquisition of the M6P recognition marker, lysosomal enzymes bind to MPRs in the TGN. The* ***receptor-lysosomal*** *enzyme complexes leave the TGN in clathrin coated vesicles which travel to and fuse with endosomes. After, the dissociation of the ligand-receptor complex due to the acidic pH (most likely in late endosomes), the MPRs recycle back to the TGN while lysosomal enzymes are transported to lysosomes. Cell surface MPRs are involved in the internalization of mannose 6-phosphate containing ligands via clathrin-coated vesicles which fuse with endosomes, from where the lysosomal enzymes are transported to lysosomes and the receptor recycles back either to the TGN or plasma membrane.*

Dissociation of ***MPR-lysosomal*** *enzyme complexes:*

From the studies of Gonalez-Noriega *et al.,* (1980) it is evident that the acidification of transport vesicles is a crucial step for the release of bound acid hydrolases from MPRs. At a pH below 5.7, ligands (acid hydrolases) are released from their receptors (MPRs) (Sahagian *et al.,* 1981). When cells are exposed to weak bases (Sando *et al.,* 1979; Hasilik and Neufeld, 1980; Myerowitz and Neufeld, 1981; Robey and Neufeld, 1982; Merion and Sly, 1983) and inophore such as monensin (Valdutiu and Rattazzi, 1980)

which lead to a rise in the pH of acidic organelles above 6 (Ohkuma and Poole, 1978), the dissociation of receptor-ligand complex is inhibited. When dissociation is inhibited, cells get rapidly depleted of the unoccupied receptors (MPRs). As a consequence, receptor-dependent functions (i.e. sorting of endogenous and endocytosis of exogenous lysosomal enzymes) have been shown to be blocked.

***Recycling of MPRs from** endosomes:*

Diaz and Pfeffer, (1998) identified a protein called as T1P47 (tail interacting protein 47) which is essential for the transport of the receptors from endosomes to the Golgi complexes, both *in vitro* and *in vivo*, by binding to the cytoplasmic domains of both the MPRs. TIP47 interacts with a Phe-Trp motif in the CD-MPR (Cation Dependent MPR) with the involvement of Rab9 GTPase (Lombardi *et al*, 1993; Diaz and Pfeffer, 1998; Carroll *et al*, 2001; Barbero *et al*, 2002). This Phe-Trp motif is essential for the diversion of CD-MPRs from lysosomes (Schweizer *et al*, 1997). Cellular depletion of TIP47 protein triggered the same phenotype as mutation of the CD-MPR Phe-Trp residues; that is, MPRs are missorted into lysosomes (Diaz *et al*, 1998). This suggests that productive binding of TIP47 to the Phe-Trp signal in the CD-MPR is needed for retrieval of MPRs from endosomes and delivery to the Golgi complex. TIP47 can bind to CI-MPR (Cation Independent MPR) despite its lack of a Phe-Trp motif. This binding is due to the hydrophobic residues (PPAPRPG) in the membrane proximal region of the CI-MPR cytoplasmic domain (Orsel *et al*, 2000). Moreover, in a recent study Miwako *et al*, (2001) demonstrated that cholesterol is required for the exit of cation independent MPR from the endosomal carrier vesicle/multivesicular bodies (ECV/MVBs) to the Golgi.

Mannose 6-phosphate dependent transport in vivo:

Mannose 6-phosphate residues in lysosomal enzymes play a crucial role in their transport by two physiologically significant ways.

(1) First, in certain cells, they are indispensable for targeting endogenous lysosomal enzymes to lysosomes. This appears especially in I-cell disease, where lysosomal enzymes lack the recognition marker, that is required for receptor mediated endocytosis (Hickman and Neufeld, 1972). This results in intracellular deficiency and extracellular accumulation of many lysosomal enzymes. The inability to synthesize the recognition marker on the lysosomal enzymes is due to the deficiency of N-acetylglucosamine 1-phosphotransferase (Bach *et al,* 1979; Hasilik and Neufeld, 1980; Hasilik *et al,* 1981; Reitman *et al,* 1981).

(2) The second function of mannose 6- phosphate residues is to mediate intercellular exchange of lysosomal enzymes. This function is clearly seen in Hunter's disease, an X-linked lysosomal disorder that is characterized by the deficiency in iduronate sulfatase (Neufeld, 1991).

MANNOSE 6-PHOSPHATE RECEPTORS:

Types of Mannose 6-phosphate receptors:

MPRs are divided into two types based on their requirement of metal ions for ligand binding or based on their molecular mass - Cation Independent MPR (CI-MPR or MPR 300) (Shangian *et al,* 1981) and Cation Dependent MPR (CD-MPR or MPR 46) (Hoflack and Kornfeld, 1985). Both the MPRs are type I integral membrane glycoproteins that constitute the family of P-type lectins, a designation given based on the unique ability of these proteins to recognize phosphorylated mannose residues (Drickamer and Taylor, 1993). CI-MPR is a multifunctional receptor that binds in addition to mannose 6-phosphorylated proteins several distinct nonglycosylated ligands, including IGF-II (Table 2).

M6P-containing ligands	Consequence of binding to the IGF-II/MPR (CI-MPR)	References
Lysosomal enzymes	Endocytosis and/or targeting to lysosomes	Kornfeld and Mellman, 1985; Kornfeld, 1992; Hille-Rehfeld, 1995; Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001.
Transforming growth factor-β precursor (TFG- β)	Proteolytic activation at the cell surface	Purchio *et al.*, 1988; Jirtle *et al.*, 1991; Dennis and Rifkin, 1991; Munger *et al.*, 1997; Godar *et al.*, 1999; Ghahary *et al.*, 1999; 2000
Leukemia inhibitory factor	Endocytosis and degradation of lysosomes	Blanchard *et al.*, 1998; 1999
Proliferin	Induction of endothelial cell migration and angiogenesis	Lee and Nathans, 1988; Volpert *et al.*, 1996; Jackson and Linzer, 1997; Groskopf *et al.*, 1997.
Thyroglobulin	Endocytosis and activation and/or degradation in lysosomes?	Herzog *et al.*, 1987; Scheel and Herzog, 1989; Lemansky and Herzog, 1992.
Renin precursor	Internalization and proteolytic activation and/or degradation in lysosomes	Faust *et al.*, 1987; Aced *et al.*, 1992; van Kesteren *et al.*, 1997; Admiraal *et al.*, 1999; Danser *et al.*, 1999; Saris *et al.*, 2001; van den Eijnden *et al.*, 2001.
Granzyme A	Targeting to lytic granules and role in apoptosis?	Griffiths and Isaaz, 1993; Burkhardt *et al.*, 2000
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; 1999; Cacia *et al.*, 1998.
CD26	Internalization and role in T cell activation	Ikushima *et al.*, 2000; Ohuma *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; 1998
Varicella-zoster viral glycoprotein I	Facilitation of viral entry into cells	Gabel *et al.*, 1989; Wang *et al.*, 2000.
NON M6P-CONTAINING LIGANDS		
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-β precursor at the cell surface; endocytosis and degradation in lysosomes.	Godar *et al.*, 1999; Nykjaer *et al.*, 1998; Nunes *et al.*, 1995.
Plasminogen	Conversion to plasmin, which can activate TGF-β precursor.	Godar *et al.*, 1999.

Table 2. Reported ligands for the CI-MPR (IGF-II/MPR) (Dahms and Hancock, 2002)

CD-MPR is known to transport lysosomal enzymes from TGN to lysosomal compartment along with CI-MPR. Unlike the CI-MPR, CD-MPR does not function in endocytosis of mannose 6-phosphate ligands.

Distribution:

MPRs are distributed over several intracellular compartments (Kornfeld & Mellman, 1989). In the *trans*-Golgi network (TGN), they mediate sorting of lysosomal enzymes from secretory proteins while on the plasma membrane they function in endocytosis of extracellular ligands. The MPRs are in rapid equilibrium between these compartments and the early/late endosomes where they release their ligands (Ludwig *et al.,* 1992). It is clear that some crucial steps in their routing require specific signals contained in their cytoplasmic domains that interact with specific cellular sorting components, some of which form the coat of clathrin-coated vesicles.

The receptors contain multiple signals in their cytoplasmic domains that mediate both their departure from and arrival at the TGN, plasma membrane, and early or late endosomes. Two serine residues (serine 2421 and 2492) found in two highly conserved regions of the carboxy terminal domain of the CI-MPR are phosphorylated *in vitro* by a casein kinase II- type enzyme present in clathrin-coated vesicles (Meresse *et al.,* 1990). These two serines are phosporylated *in vivo* when the CI-MPR leaves the TGN and/or when present in TGN derived clathrin-coated vesicles (Meresse & Hoflack 1993). This phosphorylation appears as a major albeit transient modification in the cytoplasmic tail of the CI-MPR that is subsequently delivered to early endosomes. Phosporylation of these key serines does not occur when the cell surface CI-MPR is endocytosed or when it travels through the early and late endosomes, but only occurs after it has recycled back to the TGN. Mutational studies indicate that serine 2492, as well as, an adjacent dileucine motif is required for efficient intracellular retention of lysosomal enzymes (Chen *et al.,*

1993). Recent studies indicate that the prime candidates for this role are the Golgi-localized, g-ear-containing ARF-binding proteins (GGAs) (Boman *et al,* 2000; Dell'Angelica *et al,* 2000; Hirst *et al,* 2000; Poussu *et al,* 2000). Three GGAs (GGA1, GGA2 and GGA3) have been identified in human and two in yeast (Gga1p and Gga2p). GGA proteins have a common domain organization. In the NH2-terminus, they have a VHS (Vps27/Hrs/STAM) domain, which is conserved in various proteins involved in endocytic trafficking (Lohi *et al,* 1998). Recently, Rosa Puertollano *et al,* (2001) identified highly specific interactions of acidic-cluster-dileucine signals involved in sorting of MPRs at the TGN with the VHS domain of the GGAs. Yunxiang Zhu *et al,* (2001) demonstrated that GGA2 is responsible for binding to the acidic cluster-dileucine motif in the cytoplasmic tail of the cation-independent mannose 6-phosphate receptor (CI-MPR). Receptors with mutations in this motif were defective in lysosomal enzyme sorting. The hinge domain of GGA2 binds to clathrin, suggesting that GGA2 could be a link between cargo molecules and clathrin-coated vesicle assembly. Thus, GGA2 binding to the CI-MPR is important for lysosomal enzyme targeting (Yunxian Zhu *et al,* 2001). Comparison of the crystal structures of the different GGA's reveal that the significant difference between GGA2 and GGA1, GGA3 is in the loop between helices 6 and 7, which forms part of the ligand binding pocket (Zhu *et al,* 2003).

Importance of the two receptors:

The biological function of the existence of the two MPRs in mammalian cells is an important unresolved question. Studies on the function of the CI-MPR have been greatly facilitated by the use of cells lacking this receptor. Expression studies in these cells indicate that the CI-MPR is primarily involved in intracellular retention of lysosomal enzymes (Lobel *et al,* 1989). In contrast, studies on the function of CD-MPR in lysosomal enzyme trafficking have been limited by the lack of cells devoid of this

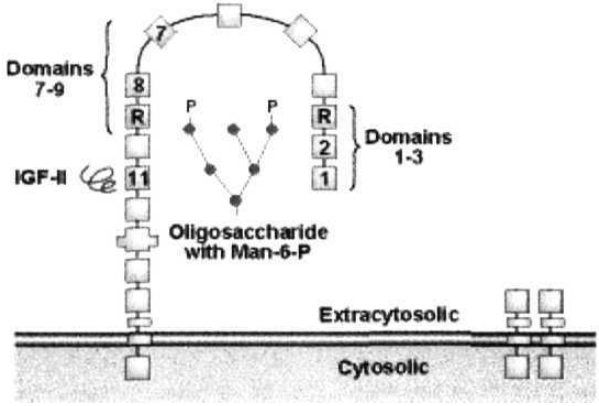
receptor. This was further supported by cDNA (encoding MPR) transfection studies in CD-MPR deficient murine tumor cells expressing MPR 46, where the hyper secretion (60-75%) of newly synthesized lysosomal enzymes was completely corrected (reduced to 3%) by over expression of CI-MPR and partially corrected (reduced to 30 - 35%) by over expression of CD-MPR (Gabel *et ai,* 1983; Kyle *et al,* 1988; Lobel *et al,* 1989). Cells lacking either the CI-MPR or the CD-MPR secrete a part of their lysosomal enzymes and as a consequence are impaired in intracellular degradation as visualized by the accumulation of undigested material in the lysosomes. Immunological studies using anti-receptor antibodies for inhibiting ligand binding to receptor in various cell types such as cells expressing both receptors and cells expressing only CD-MPR, indicated that both receptors participate in ligand binding but with different affinities (Gartung *et al,* 1985; Stein *et al,* 1987). Cells lacking both MPRs were almost completely impaired in targeting their lysosomal enzymes and also exhibited an enormous accumulation of undigested material in the cytoplasm.

Primary Structure of MPRs:

Both mannose 6-phosphate receptors have been cloned and sequenced at the cDNA level from various species (Table 3). Both receptors are integral membrane proteins with three distinct domains, the extracytoplasmic domain, the transmembrane domain and the cytoplasmic domain. Figure 3 shows a schematic representation of both receptor structures. (for reviews see Hille-Rehfeld, 1995; Pohlmann, 1996 Dahms and Hancock 2002).

	CI-MPR (MPR 300)	CD-MPR (MPR 46)
cDNA Cloning	1. Human (Morgan *et al.*, 1987; Oshima *et al.*, 1988)	1. Human (Pohlmann *et al.*, 1987)
	2. Bovine (Lobel *et al.*, 1987; Lobel *et al.*, 1988)	2. Bovine (Dahms *et al.*, 1987)
	3. Rat (MacDonald *et al.*, 1988)	3. Murine (Koester *et al.*, 1991; Ludwig *et al.*, 1992; Ma *et al.*, 1992)
	4. Mouse (Szebenyi and Rotwein, 1994)	
	5. Goat partial (Present study)	**4. Goat (Present study)**
	6. Chicken (Zhou *et al.*, 1995)	5. Chicken (Matzner *et al.*, 1996)
	7. Fish partial (Uadaya *et al.*, 2000)	

Table 3: Molecular cloning of MPR proteins from different animal species.



CI-MPR CD-MPR

Figure 3: Schematic representation of the primary structure of Mannose 6-phophate receptors (Dahms)

MPR 300 (CI-MPR):

The first purification of MPR 300 from bovine liver was achieved by Sahagian *et al*, (1981), by using the lysosomal enzyme β-galactosidase immobilized to Sepharose 4B. Later, the receptor has been isolated from a variety of tissues and cells including bovine, human, goat, chicken, fish livers and cell lines like human fibroblasts, rat hepatocytes, Chinese hamster ovary cells, chicken embryonic fibroblast cells and also from an invertebrate mollusc (Udaya Lakshmi *et al*, 1999). The cDNA of MPR 300 has been cloned from several species such as the bovine (Lobel *et al*, 1987, 1988), human (Morgan *et al*, 1987; Oshima *et al*, 1988) mouse (Lobel *et al*, 1988; Oshima *et al*, 1988), rat (MacDonald *et al*, 1988) chicken (Zhou *et al*, 1995), pig, rabbit, bat, colugo, hedgehog, ring-tailed lemur, tree shrew, opossum, red-necked wallaby, echidna and the duckbill platypus (Killian *et al*, 2001). Partial cDNA sequences of IGF-II/MPR have also been known for kangaroo (Yandell *et al*, 1999) and fish (Udaya Lakshmi *et al*, 2000). The bovine IGF-II/MPR is comprised of a 44 residue amino-terminal signal sequence, a large 2269 residue extracytoplasmic region, a single 23 residue transmembrane region, and a 163 residue carboxyl terminal cytoplasmic domain. The larger extracytoplasmic region is composed of 15 repetitive domains of similar size (average of 147 residues) that display amino acid sequence identity (14-38%). It was investigated that the repeat domain 13 from the amino terminus contains a 43 amino acid residue insertion that is similar to the sequences found in fibronectin (Hynes, 1985). This segment forms a part of the collagen-binding domain in fibronectin, but its function in the MPR 300 is not yet known.

The ligand-binding region in MPR 300 has been localized to domain 3 and 9 in the extracytoplasmic region. This was further confirmed by partial proteolysis and expression of the truncated form of the receptor. Arginine residue 435 in domain 3 and

1334 in domain 9 are essential for carbohydrate recognition (CRD) (Westland *et al.*, 1991; Dahms *et al*, 1993). These two CRDs show different binding ability to mannose 6-sulfate and mannose 6-phosphate methyl ester found on *Dictyostelium discoideum* lysosomal enzymes. The amino terminal CRD (i.e, 3rd domain) binds mannose 6-sulfate and mannose 6-phosphate methyl ester with a 14-55 fold higher affinity than the carboxyl-terminal CRD (i.e., domain 9) (Marron *et al.*, 2000), suggesting that the CI-MPR contains two functionally distinct CRDs. Though much has been investigated about the m6p binding domains in the CI-MPR, very little is known as to what functions the other repetitive domains have.

Various groups of workers have identified that IGF-II (nonglycosylated peptide hormone) can bind to mammalian CI-MPRs, such as bovine (Morgan *et al*, 1987), human (Tong *et al.*, 1988), kangaroo (Yandell *et al.*, 1999), opossum (Dahms *et al.*, 1993). In contrast to this, Kornfeld and collegues (1989) demonstrated that chicken and *Xenopus* CI-MPR fails to bind IGF-II (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989; Yang *et al.*, 1991). However, recently Mendez *et al.*, (2001) showed that the fish embryo receptor can bind IGF-11. Tong and Kornfeld (1998) showed that binding of mannose 6-phosphate to CI-MPR does not inhibit the binding of insulin like growth factor-II. Moreover, Sally *et al.*, (1999) demonstrated that β-glucoronidase stimulates internalization of ^{125}I-IGF-II. All these experiments reveal that the mannose 6-phsophate binding site and IGF-11 binding site are distinct in CI-MPR. IGF-II binding site in CI-MPR is localized in the amino terminal portion of extracytoplasmic domain 11 of bovine and human CI-MPRs (Dahms *et al.*, 1994; Schmidt *et al.*, 1995; Garmroudi *et al.*, 1996), corresponding to amino acids 1524-1590. To date, only a single residue, isoleucine 1572, in domain 11 has been implicated as being important for IGF-II binding by mutagenesis studies in which substitution of isoleucine (1572) with threonine

eliminated IGF II biding (Garmroudi *et al,* 1996; Byrd *et al,* 1999; Devi *et al,* 1999). Recent studies have provided evidence that the IGF-II binding site of the IGF-II/MPR is bipartite: the primary determinants for binding reside in domain 11 while sequence elements within domain 13 contribute a ~5 - 10 fold enhancement of the binding affinity of the receptor for IGF-II (Devi *et al.,* 1998; Grimme *et al,* 2000; Linnell *et al.,* 2001).

Nineteen potential N-linked glycosylation sites are distributed throughout the extracytoplasmic region of the CI-MPR (Lobel *et al.,* 1987), although it is unclear how many of the sites are actually glycosylated. Importantly, it has been shown that N-linked glycosylation is not required for the acquisition of mannose 6-phosphate or IGF-II binding ability by the CI-MPR. (Goldberg *et al.,* 1983; Sahagian *et al.,* 1983).

The position of the cysteine residues is highly conserved among all the repeats in the extracytoplasmic region. The presence of intramolecular disulfide bonds in the CI-MPR has been confirmed indirectly in a number of studies by the use of reducing agents (Hille -Rehfeld, 1995; Dahms and Hancock, 2002). Disulfide bond formation is predicted to be an essential early step in the proper folding of the CI-MPR.

For MPR 300, the overall homology from mouse to man was found to be 82%. Although the cytoplasmic domains show considerable variation (80% homology from mouse to human), they retained functionally important stretches of highly conserved amino acids that are involved in endocytotic sorting, internalization and G-protein binding. It also contains four highly conserved sequences that are known to be potential substrates for various protein kinases including protein kinase C, cAMP-dependent protein kinase and casein kinases I and II (MacDonald *et al.,* 1988). The receptor (CI-MPR) is known to be phosphorylated at a number of these sites (Sahagian and Neufeld, 1983; Corvera and Czech, 1985; Meresse *et al.,* 1990). Although phosphorylation of threonine, tyrosine and serine residues in the cytoplasmic tail of CI-MPR has been

observed, only the later has been studied in detail (Sahagian and Neufeld, 1983; Corvera *et al.,* 1986; 1988). The cytoplasmic tails of the bovine CI-MPR harbors three potential serine phosphorylation sites: serine 19, 85, and 156. Phosphorylation of serine 85 and 156, both of which reside in casein kinase-like motifs, has been demonstrated by *in vitro* and *in vivo* studies (Corvera *et al,* 1988; Meresse *et al,* 1990; Rosorius *et al.,* 1993). From the studies of Byrd *et al.,* (2000), it is evident that CI-MPR is capable of forming oligomeric complexes, most likely dimers, in the absence of mannose 6-phosphate ligands.

The complete genomic structure of the IGF-II/MPR has been analyzed for the mouse (Szebenyi and Rotwein, 1994) and the human (Killian and Jirtle, 1999). The IGF-II/MPR locus in mouse is chromosome 17 and human in chromosome 6. The total size of the human IGF-II/MPR gene is estimated to be 136kb and consists of 48 exons (Killian and Jirtle, 1999). Exons 1-46 encoding for the extracellular region of the IGF-II/MPR, with each of its 15 domains encoded by portions of three to five separate exons (Szebenyi and Rotwein, 1994; Killian and Jirtle, 1999).

This receptor (M6P/IGF-II or MPR 300) is the first example of a protein able to bind three different classes of ligands, that is a saccharide (M6P), a peptide (IGF-I1) and a lipid (retinoic acid) (Vidal *et al,* 2002).

MPR 46 (CD-MPR):

It occurs as a dimer in the membranes. CD-MPR was first purified and characterized from bovine liver and mouse P388D1 macrophages (Hoflack and Kornfeld, 1985). Later, the cDNA for the MPR 46 has been cloned and sequenced from several species such as human (Pohlmann *et al,* 1987), bovine (Dahms *et al,* 1987), mouse (Koster *et al,* 1991; Ma *et al.,* 1991; Ludwig *et al,* 1992), rat (Kanamori *et al,* 1998) and partially

from chicken (Matzner *et al,* 1996). The mRNA of the human MPR 46 codes for a protein of 277 amino acids consisting of four structural domains viz., a N-terminal signal sequence of 20 or 26 amino acids, an extracytoplasmic domain of 164-170 amino acids exposed at the plasma membrane or oriented to vesicle lumen, a single transmembrane domain of 20 amino acids followed by 67 amino acids of cytoplasmic domain. The short extracytoplasmic domain was shown to exhibit 14-37% homology to individual repeats of MPR 300 (Dahms *et al,* 1987; Lobel *et al,* 1988). This suggests that the two receptors may be derived from a common ancestor, in contrast to this, there is no sequence similarity among the signal sequences, transmembrane regions and the cytoplasmic domains of the two receptors.

CD-MPR is a highly glycosylated protein, the carbohydrates constituting about 20% of the total mass of the receptor. The extracytoplasmic domain of the CD-MPR contains five potential N-linked glycosylation sites (Asn-X-Ser/Thr) at positions 31, 57, 68, 81 and 87 (Dahms *et al,* 1987), four of which are used. This glycosylation is not necessary for ligand binding (Wendland *et al,* 1991; Zhang *et al,* 1993). Site directed mutagenesis experiments revealed that of the 5 glycosylation sites 31, 57 and 87 are not required for oligomerization and do not contribute to the binding affinity of the CD-MPR for p-glucuronidase. Only 154 residues in the extracellular region can autonomously fold into a fully functional carbohydrate recognition domain (Patricia *et al,* 1998).

The position of cysteine residues which are most likely involved in disulfide bond formation are well conserved within the extracytoplasmic domain of MPR 46. Studies carried out by various workers (Hille *et al.,* 1989; Li *et al,* 1990; Wendland *et al,* 1991) revealed that six cysteine residues of the extracytoplasmic region are used to form three intramolecular disulfide bonds that are essential for generating the ligand binding conformation of the CD-MPR.

MPR 46 is a highly conserved protein with 93-95% overall sequence homology from mouse to human and with completely identical amino acid sequence within the cytoplasmic domain of these species. The cytoplasmic domain contains a single casein kinase-II phosphorylation site. The gene for the human MPR 46 has been localized to chromosome 12 (Pohlmann *et al.*, 1987). The gene spans about 12kb and consists of 7 exons (Klier *et al.*, 1991). Exon 1 contains 5' untranslated sequence, exon 2 encodes the signal sequence and the beginning of the luminal domain, and exons 3-5 encode the remaining of the luminal domain. Exons 5 and 6 encode the transmembrane domain, and exons 6 and 7 encode the cytoplasmic domain (review Kornfeld, 1992).

Chemical modification studies on the human CD-MPR in the presence and absence of Man 6-P have suggested that histidine (131) and arginine (137) residues are involved in ligand binding (Stein *et al.*, 1987).

Ligand Binding Properties of MPRs:

Equilibrium dialysis experiments indicate that the CD-MPR binds one mole of the monovalent ligand mannose 6-phosphate and 0.5 mole of a diphosphorylated high mannose oligosaccharide per monomeric subunit (Tong and Kornfeld, 1989; Distler *et al,* 1991). Consequently each dimer would have two mannose 6-phosphate binding sites, both of which can be occupied by a single oligosaccharide containing two man 6-phosphate residues. On the other hand, CI-MPR binds two moles of mannose 6-phosphate or one mole of diphosphorylated oliosaccharide per monomer (Tong and Kornfeld, 1989; Distler *et al,* 1991, Tong *et al,* 1989), suggesting that only 2 of the 15 repetitive domains of the receptor may be involved in the mannose 6-phosphate ligand binding. Later it was shown that Arginine 435 in domain 3 and Arginine 1334 in domain 9 are involved for ligand binding (Dahms *et al,* 1993). In addition to this it was

also observed that the two mannose 6-phosphate binding sites are not functionally equivalent in their ligand binding affinities (Patricia *et al.*, 1998).

Ligand binding affinities of both receptors to various mannose 6-phosphate containing ligands are shown in the Table 4.

Ligand	MPR 300	MPR 46
Mannose 6-phosphate	$7\text{-}8 \times 10^{-6}$ M	$7\text{-}8 \times 10^{-6}$ M
PMP (Pentamannosyl Phosphate)	6×10^{-6} M	6×10^{-6} M
High mannose oligosaccharides with two phosphomonoesters or lysosomal enzymes	2×10^{-9} M	$7\text{-}8 \times 10^{-7}$ M
Methyl 6-phosphomannosyl diesters (from Dictyostelium discoideum)	Shows a weak Binding	No binding

Table 4: Affinity constants of MPRs to various mannose 6-phosphate containing ligands.

Multifunctional nature of MPR 300:

Apart from the transport and sorting of acid hydrolases from TGN to prelysosomal compartment, cation independent mannose 6-phosphate receptor exhibits the following unique functions:

IGF-II MPR binding to cell surface and activation:

Binding of several growth factors such as transforming growth factor beta-1 (TFG-β1) precursor and prolifirin to IGF-II/MPR on the cell surface leads to their activation. TGF-β1 is a critical cytokine that regulates the cellular differentiation and proliferation of many cell types. This molecule has been found to contain mannose 6-phosphate residues and has been found to bind to the IGF-II/MPR receptor (Purchio *et ai,* 1988; Kovacina *et al.*, 1989). Inhibition of TGF-β1 activity was observed in the presence of mannose 6-

phosphate and anti mannose 6-phosphate/IGF-II receptor antibodies in bovine aortic endothelial cell/bovine smooth muscle cell co-cultures suggesting that binding to the IGF-II/MPR receptor is required for latent TGF- β1 activation (Dennis *et al.,* 1991). In a recent study by Villevalois-Cam *et al,* (2003), it was observed that the expression of CI-MPR was increased in mid G1 phase and that hepatocyte carrying CI-MPR was a direct target for latent pro TGF β activation. Another growth factor prolifirin is also known to contain mannose 6-phosphate residues. It's binding to the IGF-II/MPR could result in its activation in endosomes or its degradation in lysosomes. Moreover, a recent study which demonstrated the ability of plasminogen and urokinase-type plasminogen activator receptor (uPAR) to bind to IGF-II/MPR at regions of the receptor distinct from the M6P binding pockets and the IGF-II binding site, suggest a plausible mode in which the binding of urokinase plasminogen activator to uPAR complexed to the IGF-II/MPR facilitates conversion of plasminogen to plasmin, which in turn proteolytically activates receptor-bound TGF-p precursor molecules (Nykjaer *et al.,* 1998; Godar *et al.,* 1999).

IGF-II/MPR in maternal diabetes:

Diabetic pregnancy is commonly associated with alterations in the IGF system in fetal tissues, the effect of maternal diabetes on IGFs and their receptors in developing fetal rat kidney was also investigated (Amri *et al.,* 1999). When diabetes was induced in pregnant rats by streptozotocin on day 0 of gestation, levels of IGF-II increased drastically throughout the nephrogenesis when compared to the control, where as IGF-I levels remained unchanged, suggesting a critical role of IGF-II in kidney development. They also observed increased production of IGF-II/MPR throughout nephrogenesis. The altered nephrogenesis in fetuses exposed to maternal diabetes may be linked to a decrease in IGF-II bioavailability Amri *et al.,*(2001).

IGF-II/MPR and its relation to Cancer:

This postulate is supported by the recent findings that the M6P/IGF-II receptor is often mutated in dysplastic liver lesions and HCCs (Hepatic Cell Cancer) in patients with or without hepatitis virus (HV) infection (De Souza *et al,* 1995; Yamada *et al,* 1997) and in breast cancer (Hankins *et al,* 1996). The IGF-II/MPR also contains a poly-G region, and it is a common mutational target in colon, gastric and endometrial tumors with mismatch repair deficiencies (Ouyang *et al,* 1997). Furthermore, in HV-infected patients, IGF-II/MPR allelic loss has already occurred in the phenotypically normal liver tissue adjacent to dysplastic lesions and HCCs (Yamada *et al,* 1997). This demonstrates that IGF-II/MPR interaction occurs early in liver carcinogenesis. It also suggests that the incidence of HCCs in HV-infected patients is significantly enhanced because the liver is repopulated with normal appearing IGF-II/MPR-mutated pre-cancerous liver cells.

There is also evidence that some chemopreventive and therapeutic agents elicit their biological effects through the IGF-II/MPR. The monoterpenes, limonene and perillys alcohol, are agents that are effective in the treatment of a variety of rodent tumors (Jirtle *et al,* 1993). Mammary tumors regressing in response to limonene have been shown to overexpress the IGF-II/MPR gene whereas, its expression is unaltered in unresponsive tumors (Jirtle *et al,* 1993). The IGF-II/MPR has also been shown to be a high affinity binding receptor for retinoic acid. Furthermore, it is required for this anticancer agent to induce cell death in human promyelocytic leukemia cells lacking the retinoid nuclear receptor (Rang *et al,* 1997). It may now be possible to diagnose patients having greatest response to these two classes of anticancer agents prior to the initiation of therapy, by determining whether the IGF-II/MPR is still functional in the tumor. Thus, the IGF-II/MPR may provide a unique target for cancer diagnosis, therapy and chemoprevention.

Evolution of Mannose 6-phosphate receptors:

No simple evolutionary picture can be expected to emerge from comparison of non-mammalian vertebrates and invertebrates with highly evolved mammals. However, recognition of elements in particular MPRs of the mammalian pattern, in non-mammalian species would provide a picture of possible conservation of fundamental mechanisms for specific sorting and targeting of lysosomal enzymes to lysosomes in the vertebrate and invertebrate lineage (Figure 4).

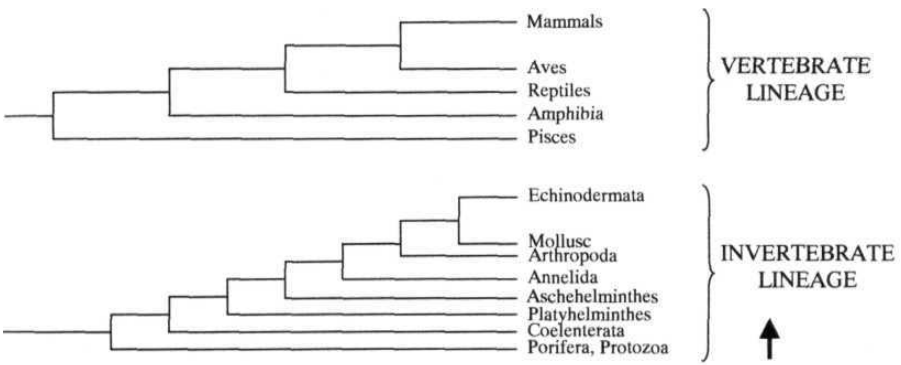


Figure 4: Vertebrate and Invertebrate Lineage with Phyla represented in the ascending order.

To date, two distinct mannose 6-phosphate receptors (MPR 300 and MPR 46) have been identified and well characterized in mammals (for review see Dahms and Hancock, 2002). The occurrence of both MPRs among the non-mammalian vertebrates such as aves (chicken), reptiles (garden lizard), amphibia (*Xenopus*), pisces (fish) and more recently in the invertebrate *Unio* has been established by biochemical and immunological methods (Matzner *et al.*, 1996; Siva Kumar *et al.*, 1997; Udaya Lakshmi *et ai*, 1999; 2000; Siva Kumar and von Figura, 2002).

So far, little information is available on the machinery used for the lysosomal enzyme sorting and targeting in non-mammalian vertebrates and invertebrates. Though the lysosomal enzymes of *Dictyostelium discoideum* were shown to contain methylated mannose 6-phosphate residue, receptors involved in mannose 6-phosphate specific sorting in *Dictyostelium* have not yet been characterized (Mehta *et al,* 1996). Sorting of lysosomal enzymes (lacking mannose 6-phosphate) in the unicellular trypanosomes is thought to occur independent of mannose 6-phosphate (Oeltmann *et al,* 1994; Heute-Perez *et al.,* 1999). On the other hand, the yeast *Saccharomyces cerevisiae* ("primitive eucaryote) is known to contain large vacuoles, which are functionally similar to the mammalian lysosomes in many ways. Two enzymes from *S.cerevisiae* have been reported to contain peripheral mannosyl phosphate and mannobiosyl phosphate in diester linkage to high mannose type oligosaccharides. The vacuolar enzyme carboxypeptidase-Y (CY) containing 4 Asn linked high mannose type oligosaccharides was found to be segregated into the vacuoles independent of mannose 6-phosphate pathway and the extracellular form of invertase enzyme also exhibited similar properties (Trimble and Maley, 1997).

Scope of the Present Investigation:

The two receptors (MPRs) have been extensively characterized from bovine and human liver. The laboratory where this work was carried out had purified two MPR proteins from goat liver tissue employing a new affinity method, Sepharose-divinyl-phosphomannan (Udaya Lakshmi and Siva Kumar, 1996).

Prior to this, very little work has been done to identify the two receptors among different animal species (non-mammalian), to study their properties and to understand the evolution of the two receptors. Work carried out by Siva Kumar *et ai,* 1997 and Udaya Lakshmi *et al.*, (2000) established the existence of mammalian homologous of MPRs among different non-mammalian vertebrates and more recently in the invertebrates. (Siva Kumar and von Figura, 2002).

The present study was envisaged to use the purified goat receptors as a mammalian model to further characterize the functions of goat receptors and also similar proteins in non-mammalian vertebrates.

Towards achieving these goals, both receptors from goat were purified in large quantities and antibodies were raised. These were used to develop an ELISA method to quantify the receptors in various tissues of the same or different animal species. The antibody to MPR 300 was used to develop an immuno affinity method for purification of the receptor in a single step.

Since goat MPR 300 was purified for the first time, it was used to study its interaction with human IGF-II. Purified chicken MPR 300 was also used to analyze this binding in view of the recent report that the fish MPR 300 binds IGF-II (Mendez *et al.*, 2001).

In order to identify the amino acids essential for ligand binding among the goat and chicken MPR 46 proteins, chemical modification studies were carried out.

To know the structural similarities of purified goat receptors to other known receptors molecular cloning studies were carried out for goat receptors. A partial cDNA clone for MPR 300 and a full-length cDNA clone for MPR 46 protein obtained, revealed that the goat receptors are structurally related to bovine and human receptors.

CHAPTER - II

Purification of MPRs from goat and chicken; Development of an ELISA and an Immuno-affinity method

INTRODUCTION:

Two mannose 6-phosphate receptors are known to be involved in sorting and transport of newly synthesized lysosomal enzymes from the TGN to the prelysosomes. According to their apparent molecular mass of 46 and 300kDa, the two receptors are designated as MPR 46 and MPR 300 respectively. They are type 1 transmembrane glycoproteins, which have recently been classed as P-type lectins. In contrast to MPR 46, 300 is a multifunctional protein. The diverging functions of MPR 46 and 300 raise the question whether the expression of the two receptors may be differentially regulated in the cells and tissues. The main objective of the present study was to purify the goat receptors, raise antibodies and develop an ELISA method to quantify MPRs in mammalian (goat) and non-mammalian vertebrates (chicken).

Polyclonal antibodies to purified goat MPR 3(K) and MPR 46 proteins were raised in two separate rabbits and using these antibodies an ELISA method was developed to quantify mannose 6-phosphate receptors from the detergent extracts of five different tissues of goat and chicken. This method allows detection of ng amounts of mannose 6-phosphate receptors (Suresh *et ai,* 2002). Additionally, a new method was developed for the purification of both MPR 3(X) and 46 proteins in a single step using immuno-affinity matrices prepared using MPR 300 specific IgG and MPR 46 peptide specific IgG coupled to Affigel-10 (Suresh and Siva Kumar, 2003).

MATERIALS:

Heating block thermostat	Bangalore Genei,, Bangalore
Pipetmann 20,200,1000µl	Glaxo, India
Spectrophotometer, UV	Spectronic GENESYS5, USA

Chemicals:

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

Imidazole	Sigma
Iodoaceticacid (IAA)	Sigma
IODO-gen	Pierce Co.
Mannose 6-phosphate (Barium salt)	Sigma
β- Mercaptoethanol	Qualigens
Methanol	Qualigens
Nonidet-40 (NP 40)	Sigma
Pansorbin cell suspension (Heat inactivated *Staphylococcus aureus* cell suspension)	Calbiochem
Phenylmethylsulfonylfluoride (PMSF)	Serva
Sepharose 6B	Pharmacia
SDS	Qualigens
TEMED	Qualigens
TCA	Qualigens
TFA	Sigma
Tris-(hydroxymethyl)-aminoethane (Tris)	Qualigens
Triton X-100	Sigma, Deisenhofen
Tween 20	Sigma, Deisenhofen

Animal tissues used in the study were procured from the local slaughterhouse and carried on ice to the laboratory and kept frozen until use.

METHODS:

*Hydrolysis of **O-Phosphonomannan** Y-2448:*

This was carried out according to Bretthauer *et al.*, (1973). 2.5gm of *O*-phosphonomannan from the yeast *Hansenula holstii* (generously provided by DR.M.E.Slodki, USDA, Peoria, IL, USA) was suspended in 50 ml of water in a screw capped bottle and left overnight for swelling. 500 mg of KC1 was added and the pH of the suspension was adjusted to 2.4 with acetic acid. The contents were then hydrolyzed in a boiling water bath at 100°C for 60 min. The suspension was cooled to room temperature and centrifuged at 10,000 rpm for 30 min to remove any insoluble material. The clear supernatant was neutralized to pH 11.0 with saturated barium hydroxide. To this, an equal volume of 95% ethanol was added and left overnight at 4°C. The precipitated phosphomannan core was collected by centrifugation at 10,000 rpm for 30 min. The pelleted phosphomannan core (PM) was dissolved in water and made acidic with acetic acid, dialyzed against water and lyophilized. This was coupled as a ligand to Sepharose as described below.

Preparation of Phosphomannan Affinity Matrix:

*Activation of Sepharose with Divinyl **sulfone:***

20 ml Sepharose 6B was washed thoroughly with distilled water on a sintered glass funnel and the wet cake was suspended in 20 ml of 0.5 M sodium carbonate/ bicarbonate buffer pH 11.0. 2.0 ml of divinyl sulfone was added and the suspension was gently shaken at room temperature for 70 min and washed thoroughly with distilled water on a sintered glass funnel.

Coupling of Phosphomannan Core to the Activated gel: Phosphomannan (PM) core obtained from the hydrolysis of *O*-phosphonomannan was coupled to the activated gel. The activated gel (20 ml) obtained above was washed with 0.5 M sodium carbonate/bicarbonate buffer pH 10.0 on a sintered glass funnel and the wet cake was suspended in 20 ml of carbonate buffer pH 10.0 containing 400 mg of phosphomannan core. Coupling was allowed to proceed in cold (4°C) for 24 hours. At the end of the coupling reaction, the gel was passed through a sintered glass funnel and the solution obtained was saved to determine the extent of binding. The gel was washed with deionised water and suspended in 0.5 M sodium bicarbonate buffer pH 8.5 containing 0.2 ml of β-mercaptoethanol and mixed at room temperature for 3 hours. The gel was finally washed with distilled water and stored at 4^0C in column buffer until further use. The extent of PM coupled to Sepharose was determined as mannose equivalents as described (Dubois *et al.,* 1956). Additional PM gels were prepared in large quantities in 20 ml batches.

Preparation of Acetone Powder:

Reagents required: (All steps were performed at 4°C).

- 0.5 M $CaCl_2$ and 1 mM $NaHCO_3$
- 4 N acetic acid
- Chilled acetone
- Diethyl ether

Acetone powder was prepared following the protocol of Distler and Jourdian (1987). Liver tissue (goat and chicken) was homogenized for about 1 min in a waring blender with 1.6 vol. of 0.5 mM $CaCl_2$ and 1 mM $NaHCO_3$ and pH was adjusted to 5.0 by drop wise addition of 4 N acetic acid. The suspension was centrifuged for 15 min at 9000 rpm and the pellet was resuspended in 2.4 vol. of the same buffer. The pH was

adjusted to 5.0 and centrifuged at 9000 rpm for 15 min. The pellet was homogenized for 1 min in a waring blender with 6 vol. of chilled (-20°C) acetone. The suspension was filtered rapidly through Whatmann 3 MM filter paper placed over a Buchner funnel. The reddish brown cake obtained was re-extracted with occasional pulverization to remove ether. The dry powder obtained was stored at -80°C until use.

Extraction of Membrane Proteins and Purification of MPRs from goat-

All operations mentioned below were performed at 4°C.

Buffers used:

- **Buffer A:** 50 mM imidazole-HCl pH 7.0, 150 raM NaCl, 0.5 mM $CaCl_2$ and 0.1 raM PMSF
- **Buffer** B: 50 mM Sodium acetate pH 4.6, 150 mM NaCl, 0.5 mM $CaCl_2$
- **Buffer** C: 50 mM imidazole-HCl pH 7.0, 5 mM Sodium β-glycerophosphate and 150 mM NaCl
- **Buffer D:** Buffer C containing 0.05% Triton X-100 and 2 mM EDTA
- **Buffer** E: Buffer C containing 0.05% Triton X-100 and 10 mM $MnCl_2$

Fresh liver tissue freed from connective tissue or the acetone powder prepared as above was homogenized with 6 vol. of buffer A in a waring blender and stirred overnight. The suspension was centrifuged at 9000 rpm for 15 min and the supernatant discarded. The pellet obtained was resuspended in 6 vol. of buffer B, homogenized and centrifuged. The pellet was suspended in 6 vol. (to the pellet weight) of buffer C, homogenized and the suspension was kept stirring in cold to which deoxycholate and Triton X-100 were added to a final concentration of 0.1% and 1% respectively. The suspension was allowed to stir overnight. The suspension was then centrifuged at 4000 rpm for 15 min. and the clear supernatant containing the membrane proteins was collected. This served as the source for purifying either MPR 300 alone or MPR 46.

For the purification of MPR 300, 2mM EDTA was added to the extract. For the purification of MPR 46, 10mM $MnCl_2$ was added. The suspension was stirred for 60 min. and centrifuged at 9000 rpm for 45 min. The clear supernatant obtained was then subjected to affinity chromatography employing appropriate buffers as given below.

Purification of MPRs by affinity chromatography:

The membrane proteins obtained above were applied on two separate Sepharose~DVS~PM gels pre-equilibrated with buffer D for the purification of goat MPR 300 alone or buffer E for the purification of goat MPR 46. The flow rate of the column was maintained at 30 ml /hr, and the gel washed extensively with the respective buffers. Elution was performed with 5 mM mannose 6-phosphate in buffer D or E. The mannose 6-phosphate eluates were pooled and dialyzed extensively against buffer D or E and concentrated using amicon concentrator. Both receptors from chicken liver were purified following the same conditions described for goat receptors using separate phosphomannan gels.

Protein Estimation:

Protein estimation was done using BCA reagent employing bovine serum albumin as a standard (1mg/ml) following the manufacturer's instructions.

BCA Reagent: 10 ml of BCA and 0.5 ml of 4% $CuSO_4$

For protein estimation aliquots of the eluates were TCA precipitated (10% TCA final concentration) for 30' on ice, centrifuged at 12000 rpm for 20'. The pellet was suspended in 500 µl of double distilled water, and mixed with 1ml of BCA reagent, incubated at 37°C for 30min. The colour developed was read at 562nm.

***SDS-Poly Acrylamide Gel** Electrophoresis:*

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

Tank buffer	:	14g glycine, 3g Tris and **1g** SDS (pH 8.3) dissolved in lliter of distilled water.
2x Sample buffer	0.5M Tris HC1 pH 6.8	2.5ml
	10% SDS	4.0ml
	100% glycerol	2.0ml
(reducing agent)	**β-mercaptoethanol**	1.0ml
	Bromophenol Blue	0.05%
	D.D.water	Volume was made upto **10ml**
Resolving gel buffer:	1.5M **Tris-HCl** pH 8.8	
Stacking gel buffer:	0.5M **Tris-HCl** pH 6.8	
Acrylamide:	30% (w/v) Acrylamide, 0.8% (w/v) N N` **methylenebisacrylamide**	

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

The amounts of reagents to be used for different percentage of resolving gel and stacking gel are shown in Table 5 and Table 6 respectively. The resolving gel reagents were mixed (APS and TEMED were added at the end), poured into sealed glass plates (mini gel) and allowed to polymerize for 30 **min** at room temperature. The stacking gel solution was poured, a comb with required number of wells was inserted and allowed to polymerize for 30 min. The wells were rinsed with water. The samples were cooked at 95^0C for 5 minutes with sample buffer mixed in 1:1 ratio, centrifuged briefly and loaded into the wells. These were overlaid with tank buffer and placed in the

electrophoretic chamber at room temperature. The samples were subjected to electrophoresis for 2-3 h at 100V.

Reagents	% of the Resolving Gel		
	7.5%	10%	12.5%
H_2O (ml)	3.650	3.025	1.875
Resolving gel buffer (ml)	1.875	1.875	1.875
10% SDS (µl)	75	75	75
APS (µl)	30-40	30-40	30-40
TEMED (µl)	20	20	20

Table 5: Reagents used for the separating gel

Reagents	Volume
Double distilled water (ml)	1.875
Stacking gel buffer (ml)	0.3125
10% SDS (µl)	25
10% APS (µl)	25
TEMED (µl)	10-15

Table 6: Reagents used for the stacking gel

Silver Staining: Blum *et al.*, (1987).

Reagents: Methanol, Ethanol, AgNC>3, Hypo, Na_2CO_3, Formaldehyde and Acetic acid.

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

1. Fixative: 50% methanol, 12% glacial acetic acid, 100 µl of formaldehyde (HCHO) were taken in a flask and the volume made up to 100ml with double distilled water. The gel was soaked for 45min to overnight. (Fixative can be stored in a brown bottle and can be reused 4-5 times).
2. The gel was transferred to 50% ethanol and incubated for 1 hour.
3. The gel was rinsed 3 times with double distilled water.
4. 40mg Hypo was dissolved in 200ml of double distilled water. The gel was soaked exactly for 1 min. in the above solution with constant shaking.
5. The gel was rinsed 3 times with double distilled water.
6. 400mg $AgNO_3$ was dissolved in 200ml of double distilled water. To this 72 µl of formaldehyde was added. The gel was soaked in the above solution for 20 min. with constant shaking.
7. The gel was rinsed 3 times with double distilled water
8. Developer: 12g of Na_2CO_3 was dissolved in 200ml of distilled water. To this 104 µl of formaldehyde was added. The gel was soaked in this solution to visualize the protein bands.
9. The gel was rinsed with double distilled water.
10. The developing reaction was stopped by the addition of 12% glacial acetic acid.

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

Raising Antibodies to the Purified Receptors:

200μg of the purified receptor (either MPR 300 or MPR 46) was acetone precipitated and the pellet was suspended in 500 μl of PBS. 500 μl of Freund's complete adjuvant was added and the suspension was subcutaneously injected into two separate rabbits. The rabbits received booster doses at the third and fifth week. The rabbits were bled by ear vein puncture and the serum collected and kept frozen at -20°C. Affinity purified MSC1 antibody is highly specific for MPR 46 protein (This antibody was raised against a synthetic peptide of the cytoplasmic tail of the human liver MPR 46 protein and was affinity purified on the immobilized peptide gel) and was generously provided by Prof.Dr.K.von Figura, Goettingen, Germany.

Affinity purification of anti-receptor antibodies on receptor affinity gel:

Goat MPR 300 protein purified on phosphomannan gel was concentrated using Amicon concentrator (PM 10 membrane) and 1mg of the receptor was coupled to 1.0ml of Affigel-10 in presence of 5mM mannose 6-phosphate following the instructions described by the manufacturer. Antiserum to purified goat MPR 300 protein was extensively dialyzed against 10mM Tris-HCl buffer pH 7.4 containing 150mM sodium chloride (column buffer) and then applied to the receptor affigel at 4°C equilibrated with the same buffer. After washing the gel extensively with the column buffer, the bound IgG was specifically eluted with 100mM glycine-HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2M Tris, analyzed on a 7.5% SDS-PAGE under non-reducing conditions and stored at 4°C.

Western blot analysis:

To detect the specificity of the antibodies, goat liver MPR 300 and MPR 46, as well as, the chicken liver MPR 300 and MPR 46 that were affinity purified on phosphomannan gel were separated on a 10% SDS-PAGE and the proteins transferred to a nitrocellulose membrane. The membrane was incubated with affinity-purified goat MPR 300 IgG (10 μg) to detect goat and chicken MPR 300 proteins. To detect the goat and chicken MPR 46 proteins, antiserum to purified goat MPR 46 protein (1:1000 dilution) and the affinity purified MSC1 antibody (8 μg) were used as the primary antibodies. The receptor bands were finally detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate BCIP/NBT (Bangalore Genei, Bangalore).

DEVELOPMENTOF AN ELISA METHOD TO QUANTIFY THE MPRs:

Extraction of membrane proteins from the tissues:

1g of each tissue was processed to get the membrane proteins as described (Wenk *et al.*, 1991). 1ml of 100mM sodium acetate buffer pH 6.0 containing 0.2M NaCl, 5mM iodoacetamide, 1mM EDTA and 1mM PMSF was added to lg of the tissue that has been minced well. The suspension was thoroughly homogenized and centrifuged for 40 min at 40000rpm in an ultracentrifuge and the soluble extract was discarded. The pellet was then extracted with 50mM imidazole-HCl buffer pH 7.0 containing protease inhibitor (lmM PMSF), lmM EDTA and 0.5% Triton X-100 and incubated on ice for 15 minutes. The suspension was re-centrifuged in an ultracentrifuge as above to get the clear membrane extract. The pellet was re-extracted one more time to obtain membrane proteins. Protein concentration in the combined extracts was determined by Peterson's (1977) method using BSA as standard.

Protein estimation by Peterson's method: Peterson, (1977)

Reagents:

1. BSA standard : 1mg/ml in double distilled water, for long term storage at -20 C.

2. DOC (Sodiumdesoxycholate) : 30mg/20ml of double distilled water. Prepared freshly before use.

3. TCA (Tricholoro acetic acid) : 72%, stored at 4^0C.

4. CTC (Cu-Tartarate-Carbonate): 0.1g $CuSO_4$ x $5H_2O$ and 0.2g Na-K-Tartarate, dissolve in approx. 60ml of double distilled water; to this 10g Na_2CO_3 was added and the volume made up to 100ml with double distilled water.

5. NaOH : 0.8M.

6. SDS : 10% (at least 99% pure SDS, not electrophoresis grade (95%)).

7. Reagent A : CTC : DD H_2O : 0.8M NaOH : 10% SDS

Mix following ratio : 1 + 1 + 1 +1

8. Reagent B : Folin-Reagent (dilute 1+4 with D.D.H_2O).

Procedure:

BSA 0/2/4/6/8 µg in 500 µl of double distilled water was taken as standard and following components were added sequentially.

50 µl of DOC was added, vortexed and incubated for 10 min at RT.

50 µl of TCA was added, vortexed and incubated for 5min on ice and centrifuged at 10000 rpm for 5 min at 4°C. The supernatant was removed carefully and the pellet was dissolved in 300 µl of double distilled water. The following components were added to this sequentially.

300 µl of Reagent A was added first, vortexed and incubated for 10 min at RT.

150 µl of Reagent B was added, vortexed and incubated for 30 to 120min at RT with blank. The colour developed was read at 750nm.

ELISA Method for the quantification of the receptors:

Affinity purified antibodies against MPR proteins were adsorbed on to microtiter wells of an ELISA plate (96 wells) for 4 h at 37°C (250ng of affinity purified goat MPR 300 antibody or 1 µg of affinity purified MSC1 antibody in 50 µl of 25mM Tris-HCl, pH 7.0). The wells were washed with 200 µl of 25mM Tris-HCl buffer pH 7.0 and incubated overnight at 4°C/ 1 h at room temperature with 200 µl of buffer C (5% lipid free milk powder, 0.05% Triton X-100, 10mM sodium phosphate, 150mM NaCl pH 7.4). 50 µl of the receptor-containing solution (0.5-10ng) of purified MPR 300 or MPR 46 from goat liver or different tissue extracts of goat and chicken, (0.5-5.0µg concentration diluted in Buffer C; quantitation of protein was done according to Peterson) was bound for 2.5h at 37°C. The wells were washed 4 times with 200 µl of buffer D (0.05% Triton X-100, 10mM sodium phosphate, 150mM NaCl pH 7.4) followed by incubation with 200 µl of buffer C for 30 min at 37°C. Subsequently, 50 µl of diluted rabbit antiserum against M6P receptors (dilution in buffer C, 10^{-3} for MPR 300 antiserum or 10^{-2} for MPR 46 antiserum) was added and the plate incubated for 1h at 37°C. After washing 4 times with buffer D, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:2000 in buffer C) was added and incubated for 1 h at 37°C. The wells were washed 4 times with buffer D and one time with 200 µl of buffer E (0.1 M Tris-HCl, 0.1 M NaCl, 2mM $MgCl_2$, pH 9.5). The colour was developed with p-nitrophenyl phosphate (1mg/ml in buffer E) for 10 to 20 min at room temperature and the absorbance measured at 405nm in a microplate ELISA reader.

Development of an Immuno-affinity method for the purification of MPR proteins:

Immuno-affinity purification of MPR 300:

(a) Preparation of goat MPR 300 affigel:

Goat MPR 300 protein (1mg) purified by phosphomannan affinity chromatography was coupled to about one ml of affigel-10 (BioRad) according to the manufacturer's instructions.

(b) Purification of goat MPR 300 specific IgG:

Goat MPR 300 specific IgG was purified from the antiserum obtained for goat MPR 300 protein and applied on goat MPR 300 affigel described in previous sections.

(c) **Preparation of goat MPR 300 IgG affigel:**

MPR 300 specific IgG (2mg) was coupled to 1ml of affigel-10 as described above.

(d) Purification of goat MPR 300:

All operations were performed at 4°C. Total membrane proteins were obtained as described Maomi *et al.*, (1989). Fresh/Frozen goat liver (100g) was diced, and homogenized for 1 min with the help of a homogenizer in 200ml of a solution containing 0.1N acetic acid, and 0.1M NaH_2PO4. The homogenate was centrifuged for 15 min at 10,000g. The pellet was washed once with 350 ml of distilled water and the suspension re-centrifuged at the same speed. The pellet was resuspended in 750 ml of imidazole-HCl buffer pH 7.0 containing 0.4 M KCl and 1% Triton X-100. The suspension was stirred for 60 min and then centrifuged for 60 min at 22,000g (Heraues, Biofuge, Germany). The pH of the supernatant was adjusted to 6.5 with 2 N acetic acid

and immediately subjected to immuno-affinity chromatography on a column (containing goat MPR 300 IgG Affigel) equilibrated with imidazole-HCl buffer pH 6.5, containing 150mM NaCl, 0.05% Triton X-100 and 0.02% NaN_3 (column buffer). The gel was washed extensively with the column buffer. Finally bound protein was eluted with 100mM glycine-HCl buffer pH 2.65. An aliquot of the protein was TCA precipitated, and subjected to SDS-PAGE analysis (silver staining), as well as, for western blotting.

Immuno-affinity purification of goat MPR 46 protein:

Peptides 237 (ADGCDFVCRSKPRBVPA) and 259 (GDDZLGEESEERDDHLLP) corresponding to distinct regions of the cytoplasmic tail of human liver MPR 46 were synthesized, purified and were generously provided by Prof.Dr.K.von Figura, Goettingen, Germany. These were conjugated to hemocyanin and the conjugate was used to raise antibodies in rabbits as described by Siva Kumar *et al.*, (1991). Peptide specific antibodies were then purified on the respective peptide affinity gels. Peptide specific IgGs were then coupled separately to affigel-10. These gels were available in the laboratory.

For the purification of the goat MPR 46 protein, total membrane extracts were prepared from the acetone powder of the goat liver tissue as described in the earlier section. This was applied to the peptide affigels equilibrated with Tris-HCl buffer pH 8.0 containing 0.01% Triton X-100. The gels were extensively washed with the same buffer and the bound protein was eluted using lOOmM glycine-HCl buffer pH. 2.65. The protein eluted was neutralized and analyzed by SDS-PAGE (Laemmli, 1970) and the MPR 46 receptor identified by silver staining. In order to further characterize the binding of the goat MPR 46 protein to the peptide IgG gel, 2μg of purified goat MPR 46 protein was

iodinated with 100μCi of $Na^{125}I$ using IODO-gen to a specific activity of $1x10^7$cpm/μg as described below.

Iodination of purified goat MPR 46 protein: Fraker and Speck (1978)

1. 20mM Borate buffer pH 8.0 containing 0.05% Triton X-100 and 2mM mannose 6-phosphate.
2. IODO-gen (1,3,4,6-tetrachloro–3a,6a-diphenylglycoluril) solution: 0.8 mg of Iodogen (M.W 432.06) was directly weighed into an eppendorf tube, dissolved in 577μl of dichloromethane. 200μl of it was transferred to another tube and diluted 1-3 times (400μl) to get a final concentration of 1mM. (Iodogen rapidly initiates iodination, the thin film of iodogen on the reaction tubes minimizes the direct exposure of the protein to the oxidant).
3. IODO-gen tubes: 50-80μl of iodogen solution was uniformly coated under Nitrogen atmosphere at the bottom of a conical glass centrifuge tube.
4. Column buffer: 1mg/ml Potassium iodide, 0.05% BSA, 0.05% Triton X-100 in 10mM phosphate buffer saline pH 7.4.

The mannose 6-phosphate eluate (goat MPR 46) was concentrated in an Amicon concentrator (10 kDa cut off membrane) and 2 μg of MPR 46 protein was then acetone precipitated with chilled (-20°C) acetone (to a final concentration of 80%), incubated overnight at -20°C and centrifuged at 14000 rpm for 10min. The pellet was air dried by leaving the tubes on ice and later suspended in 60 μl of borate buffer. The sample was centrifuged briefly and the clear supernatant was transferred to a fresh conical glass tube numbered as tube 1. To this 1 μl (100 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 transferred to tube 2 (IODO-gen tube) and incubated with rotation on ice for 8 min. The reaction was stopped by transferring the contents to tube 3 (conical glass tube) containing 10 µl of 20mM β–mercaptoethanol. Tube 2 was rinsed with 140 µl of column buffer and transferred to tube 3. Additional 300 µl of column buffer was added to tube 3 to make up the volume to 500µl.

The iodinated protein fraction was separated from the free iodine by passing the sample over a 5 ml gel filtration G-25 column pre-equilibrated with 20 ml of column buffer. The sample was applied on the gel and 10 fractions each of 0.5 ml were collected in eppendorf tubes placed in the lead aluminum metal holder. 2 ul from each of the tube was transferred to fresh eppendorf tubes numbered from 1- 10 and counted for 1 min in a y-ray counter (Figure 5).

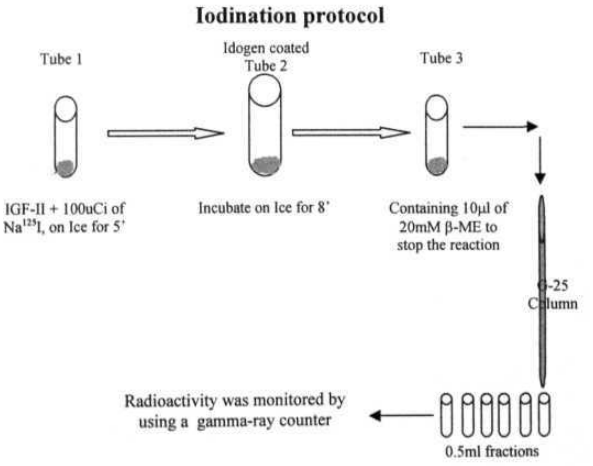


Figure 5: Schematic representation for iodination protocol.

Immunoprecipitation of iodinated goat MPR 46 protein:

50000 cpm of radiolabeled goat MPR 46 protein was applied on each of the peptide-affigel columns containing the respective specific IgG's equilibrated with Tris-HCl buffer pH. 7.4 containing 150mM NaCl. After washing the gel with the same buffer, bound radioactivity could be specifically eluted using 100mM glycine-HCl buffer, pH 2.65. In a separate experiment 50000 cpm of the iodinated goat MPR 46 protein was incubated with different concentrations of affinity purified 237 peptide IgG (30-65μg) in a total volume of 50μl of PBS in 0.02% Tween 20. After an overnight incubation in cold, the samples were treated with 20μl of pansorbin (Calbiochem) for 1 hour at 4°C and centrifuged. Pansorbin pellets were washed with PBS-Tween and the specific radioactivity bound to the pellets were counted using a γ-ray counter. Pellets were then cooked with SDS sample buffer and the clear supernatants was subjected to 10% SDS-PAGE analysis. These studies were done only for 237 peptide specific antibody.

RESULTS:

Affinity purification of goat and chicken MPR proteins:

The membrane extracts from the goat and chicken liver tissues were subjected to affinity chromatography on PM-Sepharose gel in the presence of 2mM EDTA for the purification of **MPR** 300 alone. After washing the gel extensively, the bound protein was eluted with the column buffer supplemented with 5mM mannose 6-phosphate. After depleting **MPR** 3(X) from the membrane extract, the flow through of PM-Sepharose gel was dialyzed extensively against the column buffer in the presence of $MnCl_2$ at a final concentration of 10mM and applied on PM-Sepharose gel, pre-equilibrated with column buffer containing lOmM $MnCl_2$ for the purification of **MPR** 46. After washing the gel extensively, the bound protein was eluted with the column buffer supplemented with 5mM mannose 6-phospate. An aliquot of the eluted fractions were electrophoresed on 10% SDS-PAGE (Figure 6). The yields of MPRs (MPR 300 and **MPR** 46) were about 1mg from 1kg of fresh liver tissue or 100g of acetone powder.

Affinity purification of MPR 300 specific IgG on receptor affigel:

Purified goat **MPR** 300 protein (lmg) was coupled to Affigel-10 (1ml) according to the manufacturer's instructions. Antiserum obtained for goat MPR 300 was dialysed against TBS and applied on goat MPR 300 affigel. After extensive washing, specific elution was performed with 100mM glycine-HCl pH 2.65, one ml fractions were collected and A_{280} was measured (Figure 7A). To check the purity of **MPR** 300 specific IgG, an aliquot of the eluate was subjected to 7.5% SDS-PAGE under non-reducing conditions (Figure 7B).

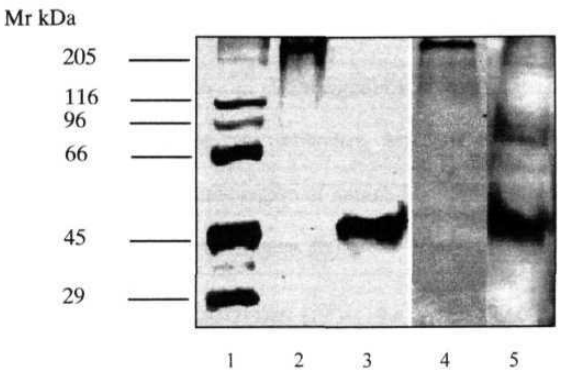


Figure 6

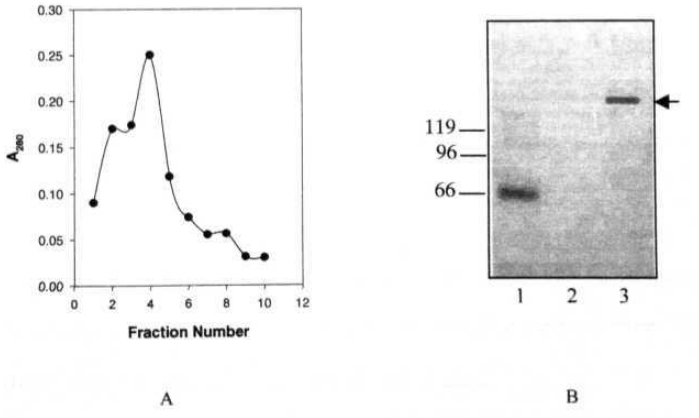


Figure 7

Western blot analysis:

Affinity purified goat and chicken MPRs were subjected to Western blot analysis as described under methods. Affinity purified goat MPR 300 antibody can specifically detect goat MPR 300 protein and also cross-reacts with chicken MPR 300 (Figure 8A). Similarly, goat MPR 46 antibody can also recognize both the goat and chicken MPR 46 proteins. In addition to these two antibodies, MSC1 antibody (peptide synthetic antibody, raised in the cytoplasmic region of human MPR 46) also recognizes both the goat and chicken MPR 46 proteins (Figure 8B).

QUANTIFICATION OF RECEPTORS BY ELISA:

Schematic diagram for an ELISA method developed in this study could be seen in Figure 9. ELISA was performed as described under methods. In order to establish precisely the concentration of the receptor that could be detected by the antibodies developed in the ELISA, the initial ELISA was carried out employing purified goat MPR 300 and MPR 46 protein. It is clear that the level of detection of the MPR 300 and MPR 46 lies in the range of l-10ng. (Figure 10). Using these standard graphs, the receptor concentration was quantified by ELISA employing the total membrane extract obtained from the liver tissue of goat (mammalian vertebrate) and chicken (non-mammalian vertebrate). With an increase in the protein concentration, there was a linear increase in the detectable range of MPR 300 and MPR 46 receptors for both species (Figure 11).

Having established the method developed which can be used to quantify the receptors in the liver tissue, we analyzed the levels of both receptor proteins from different tissues (heart, spleen, kidney and brain) of goat and chicken.

Figure 8: *Western blot analysis of goat and chicken MPRs. (A) Lanes 1 and 2 represent goat and chicken MPR 300 proteins respectively detected using affinity purified anti-goat MPR 300 antibody. The arrow indicates the position of the MPR 300 protein. (B) Purified goat MPR 46 (lanes 1 and 2) and chicken MPR 46 (lanes 3 and 4). Lanes 1 and 3 were detected using anti-goat MPR 46 antiserum and lanes 2 and 4 were detected using affinity-purified MSC1 antibody. Arrow indicates the position of the MPR 46 protein.*

Figure 9: *Schematic diagram for the ELISA method.*

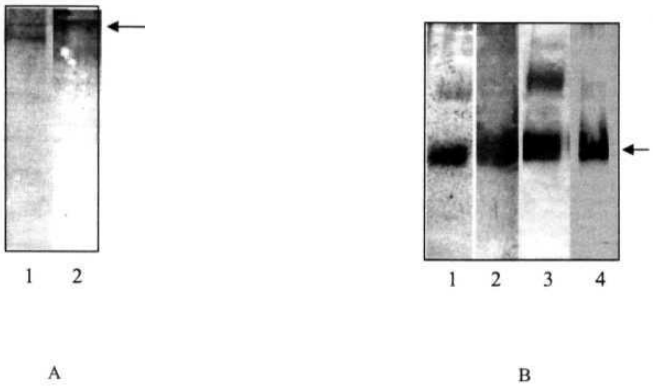


Figure 8

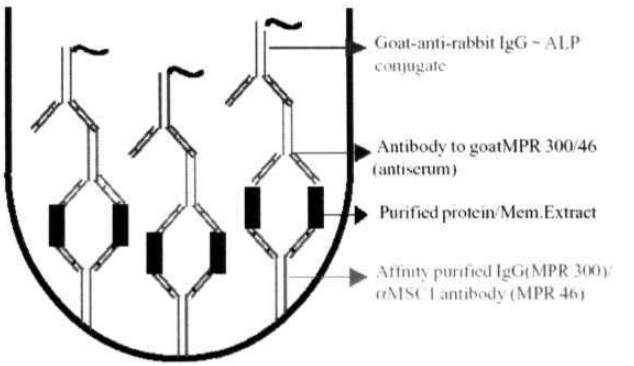


Figure 9

Figure *10:Standard graphs for the ELISA method designed using purified goat MPR proteins. The standard curve obtained with purified goat MPR 300 (a) and MPR 46 (b). The ELISA was carried out as described under materials and methods.*

Figure 11:Standard *graphs for the ELISA method performed using membrane extracts of the liver tissues from the goat and chicken. ELISA performed to detect the MPR proteins in the liver membrane extracts of goat (a and b) and chicken (c and d). (a) and (c) represent MPR 300, (b) and (d) represent MPR 46. Fifty microliters of the membrane extract at varied protein concentration was incubated with 250ng of affinity-purified anti-goat MPR 300 antibody or with I ug of affinity purified MSCl antibody. The assay was performed as described under materials and methods.*

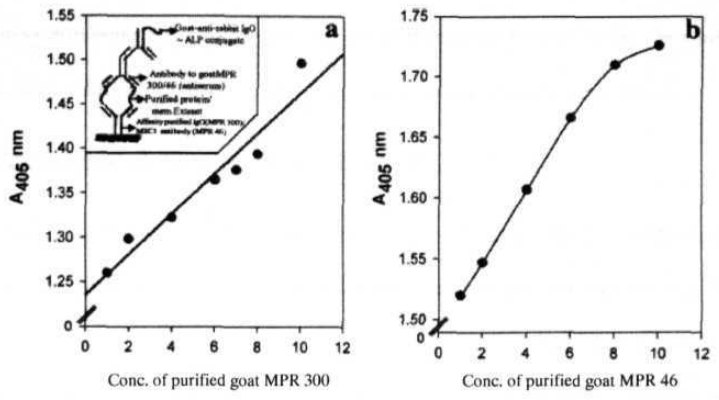


Figure 10

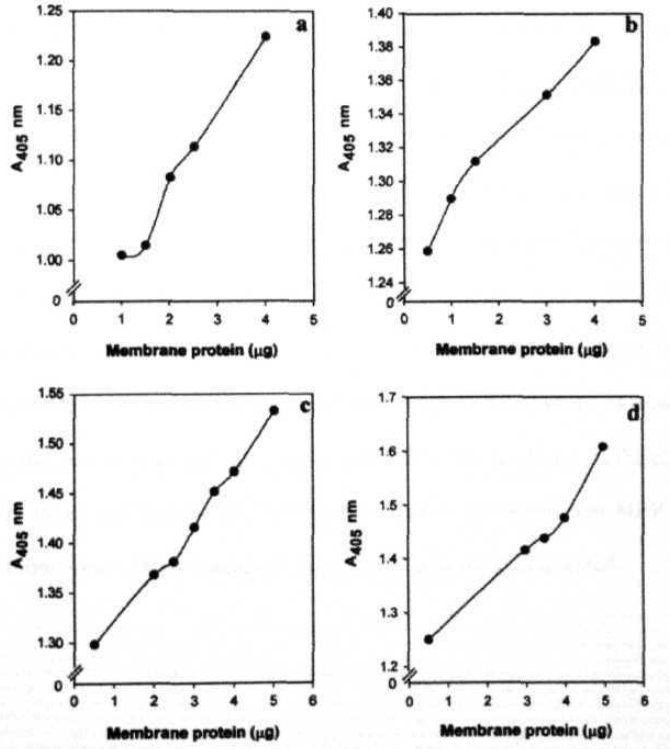


Figure 11

Comparison of the levels of MPR 300 and MPR 46 in different tissues of goat and chicken indicate that their levels are variable (Figure 12). However, the levels of MPR 46 among different tissues of chicken are higher than those found of goat. In addition to this, both MPRs are abundant in the liver tissue compared to the other tissues.

Development of an Immuno-affinity method:

As a first step towards developing an immuno-affinity matrix for the purification of mannose 6-phosphate receptor proteins, polyclonal antibodies to the purified goat MPR 300 and peptide specific antibodies corresponding to the distinct regions of the cytoplasmic tail of the human MPR 46 protein were raised in rabbits as described under methods.

The MPR 300 specific IgG could be coupled to affigel at a concentration of 1mg/ml, and the peptide specific IgGs for MPR 46 at a concentration of 5.5mg (peptide 237) and 2.0mg (peptide 259) per ml of affigel. When membrane extracts of the goat liver were passed through the MPR 300 IgG gel as described under methods, the receptor was found to bind on the gel and could be specifically eluted using 100mM glycine-HCl buffer pH 2.65. An aliquot of the eluted fraction was electrophoresed on 10% SDS-PAGE (The receptor purified on PM-Sepharose gel served as a control) (Figure 13A). 140 μg of goat MPR 300 protein was obtained from 100g of liver tissue. In order to confirm that this protein is indeed the putative goat MPR 300, an aliquot of the eluate was electrophoresed and subjected to Western blot analysis using anti-goat MRP 300 antiserum. From Figure 13B it is apparent that the protein is the goat receptor.

Figure 12: *Quantification of Mannose 6-phosphate receptor (MPR 300 and MPR 46) proteins from various tissues of goat and chicken. The ELISA was performed as described under materials and methods.*

Figure *13:(A) 10% SDS-PAGE of MPR 300. Lane 1, standard molecular weight markers; lane 2, goat MPR 300 purified on immuno-affinity gel; lane 3, goat MPR 300 purified on PM gel (the arrow indicates the position of the receptor).* **(B)** *An immuno blot analysis of the goat MPR 300 purified on immuno-affinity gel (lane 1), purified on PM gel (lane 2) ("possible proteolytic products).*

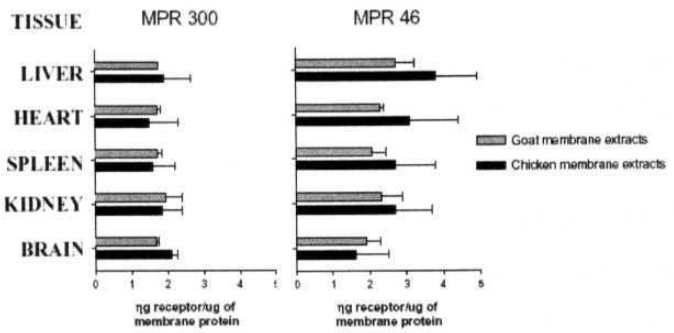


Figure 12

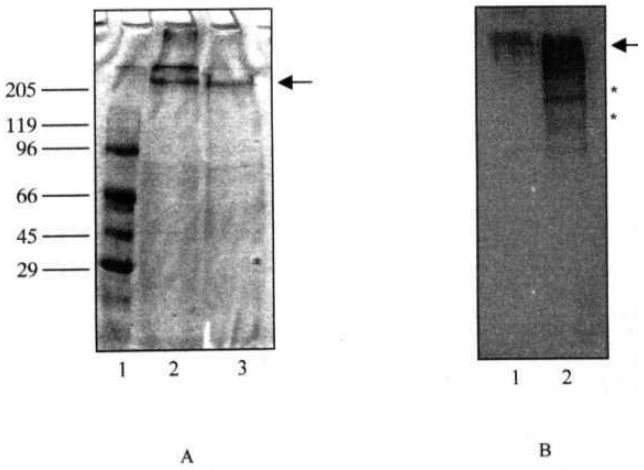


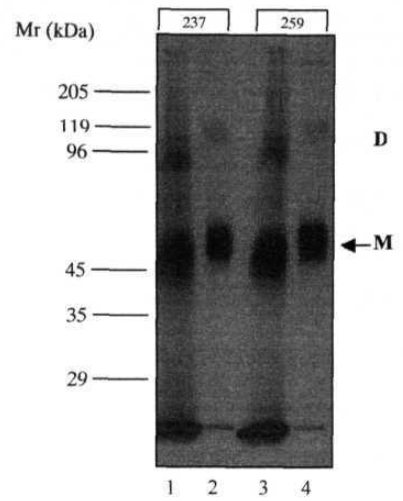
Figure 13

When the total liver membrane extracts from goat were applied on either of the peptide specific IgG gels, bound MPR 46 protein could be eluted with KK)mM glycine - HC1 buffer pH 2.65. In order to further characterize the binding of the goat **MPR** 46 to either of the affinity gels, the receptor eluted from the 237 peptide specific IgG gel was concentrated and an aliquot of the same was acetone precipitated and radioiodinated.

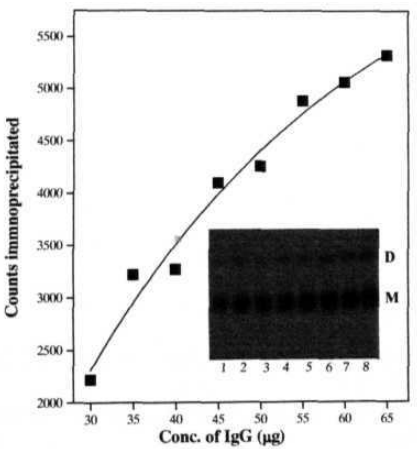
When 50,000 cpm of the radioiodinated goat **MPR** 46 receptor was applied to 0.2ml of the 237 and 259 IgG gels, 7000 cpm of the goat receptor could be specifically eluted from the gels using glycine-HCl buffer pH 2.65 (the counts could be completely TCA precipitated). A fraction of the receptor also appeared in the unbound fraction. Additionally, the appearance of the SDS-resistant dimer of the goat receptor (MPR 46) in addition to the monomer in the eluates of both gels confirms that the receptor bound to the gels (Figure 14A).

In an additional experiment, when 50,000 cpm of radioiodinated MPR 46 was incubated with different concentrations of the affinity purified 237 peptide specific IgG (30-65 μg), binding of the receptor to the IgG was found to be linear (Figure 14B).

Figure 14:*(A) 10% SDS-PAGE of the iodinated goat MPR 46 on 237 and 259 peptide affigels (lane 1 and 3, unbound fractions and lanes 2 and 4, elutions). M and D denote the monomer and SDS-resistant dimer of MPR 46. (B) Immuno precipitation of the 237 peptide specific IgG using iodinated goat MPR 46 protein. Inset (lanes 1-8 correspond to the concentration of IgG taken, details are given under materials and methods) shows the SDS-PAGE of the precipitated receptor. M and D denote the monomer and the dimer form of the receptor specifically immunoprecipitated using the 237 IgG.*


Mr (kDa)
237
259
205
119
96
45
35
29
D
M
1 2 3 4

A


Counts immunoprecipitated
5500
5000
4500
4000
3500
3000
2500
2000
30 35 40 45 50 55 60 65
Conc. of IgG (µg)
D
M
1 2 3 4 5 6 7 8

B

Figure 14

DISCUSSION:

Though mannose 6-phosphate receptors have been extensively characterized among mammals, very little is known about the evolution of these interesting proteins. Work carried out in the past couple of years established that mammalian homologous of MPR proteins are present in the liver tissues and in specific cell types of different non-mammalian vertebrates (Siva Kumar *et al.,* 1999; Udaya Lakshmi *et al.,* 2000). A recent study further demonstrated that both MPR proteins are also present in the invertebrate molluscs (Siva Kumar and von Figura 2002). Although an antibody to goat MPR 300 protein cross-reacts with homologous protein in different species, and an MSC1 antibody (raised against a synthetic peptide in the region of cytoplasmic tail of human MPR 46, provided by Prof. von Figura, Goettingen, Germany) cross-reacts with MPR 46 among different animal species, very little is known about the tissue distribution and functions of the non-mammalian MPRs and invertebrate MPRs.

In the present study, antibodies were raised to purified goat MPR proteins. Using these antibodies and the MSC1 antibody specific to MPR 46, an ELISA method was developed. Due to the high sensitivity of the assay, μg amounts of tissue protein is sufficient for the quantification of either of the receptors from different tissues of goat and chicken. The results obtained clearly showed that the steady state concentration of MPR 46 and MPR 300 vary among different tissues from goat and chicken and are possibly regulated independent of each other. Wenk *et al.,* (1991) have also found that the expression levels of the receptors among different human tissues and cell lines also vary. This method can be useful to quantify the mannose 6-phosphate receptors during the developmental stages of a particular animal species.

In order to understand the structure and function of MPR proteins in non-mammalian vertebrates, it is essential to obtain highly pure receptor preparations in large quantities.

Due to the availability of the antibodies for the goat MPR 300 and MPR 46 proteins, in the present study we explored the possibility to prepare immuno-affinity matrices that will have good potential to purify the receptors in a single step from the membrane extracts of liver tissues. To our knowledge there have been no published information on the immuno-affinity purification of the mannose 6-phosphate receptors. This study provides the first report on this aspect.

CHAPTER-III

Chemical modification studies of goat and chicken MPR 46 proteins

INTRODUCTION:

The role of specific arginine and histidine residues in the ligand binding of human and bovine MPR 46 proteins has been well characterized. However, no information is available as to which amino acids are essential for ligand binding in goat and chicken MPR 46 proteins. In the present study, since the goat and chicken liver MPR 46 proteins were purified, an attempt was made to chemically modify histidine and arginine residues in these proteins to study the effect of this modification on PM gel binding.

MATERIALS:

Arginine	LOBA Chemie
DEPC (Diethyl pyrocarobonate)	Sigma
Glucose 6-phosphate	Sigma
Histidine	LOBA Chemie
Mannose 6-phosphate	Sigma
p-Hydroxyphenylglyoxal	Pierce, Rockford, USA.
Sodium phosphate	Qualigens
Sodium pyrophosphate	Qualigens

METHODS:

Goat and chicken MPR 46 proteins were purified by phosphomannan affinity chromatography as described in the earlier chapter. Purified receptors were chemically modified using group specific reagents as described below.

Histidine modification:

This was performed according to Stein *et al.*, (1987) with minor modifications. Protein samples (~40 μg) were acetone precipitated (80%) over night. The pellet was collected by centrifugation and suspended in 200 μl of 50mM sodium phosphate buffer, pH 7.0. Diethyl pyrocarbonate was added to a final concentration of 1mM, at every 15min interval in the presence or absence of glucose 6-phosphate (5mM) or mannose 6-phosphate (5mM). The reaction mixture was incubated at 20°C for 1 hour. The modification reaction was stopped by the addition of an excess amount of histidine. The unreacted reagent was removed from the reaction mixture by dialysis against column buffer (50mM imidazole, 5mM sodium β-glycero phosphate, 150mM NaCl, 0.05% Triton X-100) and the modified protein was applied on PM-Sepharose gel (0.5ml). After washing the gel extensively with column buffer, the gel was eluted with column buffer containing 5mM glucose 6-phosphate (2 column volumes) followed by 5mM mannose 6-phosphate (2 column volumes). The wash and eluted fractions were analyzed on 10% SDS-PAGE.

Arginine modification:

This was performed according to Yamasaki (1980) with minor modifications. The receptor that is acetone precipitated as described for histidine modification was suspended in 200 μl of 0.1M sodium pyrophosphate buffer, pH 9.0. To this p-hydroxyphenylglyoxal was added to a final concentration of 2:1 (protein : reagent) in

the presence or absence of glucose 6-phosphate (5mM) or mannose 6-phosphate (5mM), at an interval of every 15min. The reaction mixture was incubated at 25°C for 1 hour. The modification reaction was stopped by the addition of an excess amount of arginine. The unreacted reagent was removed from the reaction mixture by dialysis against the column buffer and the modified protein was applied on PM-sepharose gel and processed as described for the histidine modified sample.

RESULTS:

Modification of histidine residues in purified goat MPR 46 protein:

The effect of modification of histidine residues on binding to PM-Sepharose gel was analyzed. The unmodified goat MPR 46 protein (control) was applied on PM-Sepharose gel and processed as described under methods and the extent of binding was analyzed by SDS-PAGE and densitometry (Figure 15A). When histidine was modified and the protein was applied on the gel, most of the goat MPR 46 protein could be seen in the unbound fraction and only 8% of the protein was bound to the gel which could be specifically eluted with 5mM mannose 6-phosphate in the column buffer (Figure 15B). But, when the modification was performed with pre-incubation of the protein either with 5mM glucose 6-phosphate or 5mM mannose 6-phosphate, 14% (Figure 15C) and 68% (Figure 15D) of the protein respectively could be bound to the gel which could be specifically eluted with 5mM mannose 6-phosphate in column buffer.

Modification of arginine residues in purified goat MPR 46 protein:

The effect of modification of arginine residues on binding to PM-Sepharose gel was analyzed as described for histidine residues. When arginine was modified and the protein was applied on the gel, most of the protein could be seen in the unbound fraction and only <5% of the protein was bound to the gel which could be specifically eluted with 5mM mannose 6-phosphate in column buffer (Figure 16B). But, when the modification was performed with pre-incubation of the protein either with 5mM glucose 6-phosphate or 5mM mannose 6-phosphate, 27% (Figure 16C) and 48% (Figure 16D) of the protein respectively could be bound to the gel which could be specifically eluted with 5mM mannose 6-phosphate in the column buffer.

Figure 15: *Modification of histidine residues in the purified goat MPR 46 protein. (A) Native MPR 46 protein (without modification, Control).Histidine modification was performed without (B) or with 5mM glucose 6-phosphate (C) or with 5mM mannose 6-phosphate (D).*

Lanes 1, unbound; 2, wash; 3, glucose 6-phosphate elute; 4, mannose 6-phosphate eluate; 6, molecular weight markers.

% mentioned on the left side of the gel picture is donoted % of MPR 46 eluted from the PM gel with 5mM mannose 6-phosphate.

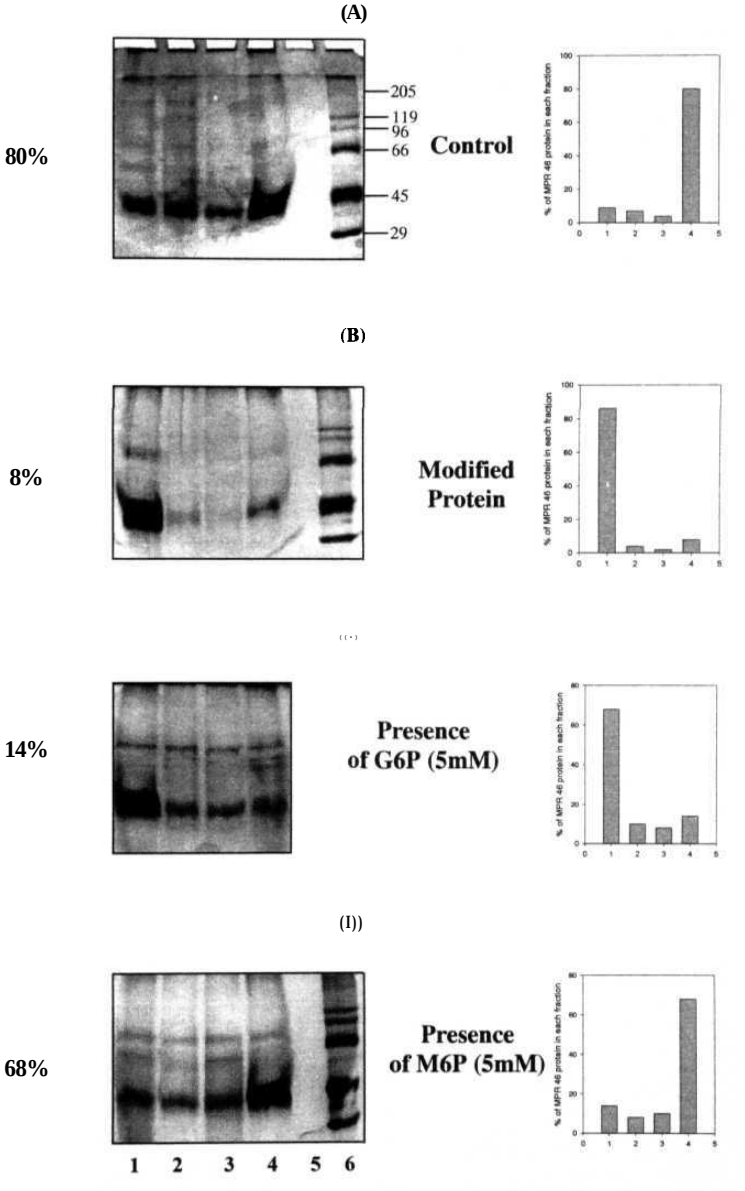

(A)
80%
205
119
96
66
45
29
Control
% of MPR 46 protein in each fraction
(B)
8%
Modified
Protein
% of MPR 46 protein in each fraction
14%
Presence
of G6P (5mM)
% of MPR 46 protein in each fraction
(I))
68%
Presence
of M6P (5mM)
% of MPR 46 protein in each fraction
1 2 3 4 5 6

Figure 15

Figure 16: *Modification of arginine residues in the purified goat MPR 46 protein.(A) Native MPR 46 protein (without modification,* ***Control****). Arginine modification was performed without* ***(B)*** *or with* ***5mM*** *glucose 6-phospate (C) or with 5mM mannose 6-phosphate (D).*

Lanes 1, unbound; 2, wash; 3, glucose 6-phosphate eluate; 4, mannose 6-phosphate eluate; 6, molecular weight markers.

% mentioned on the left side of the gel picture is donoted % of MPR 46 eluted from the PM gel with 5mM mannose 6-phosphate.

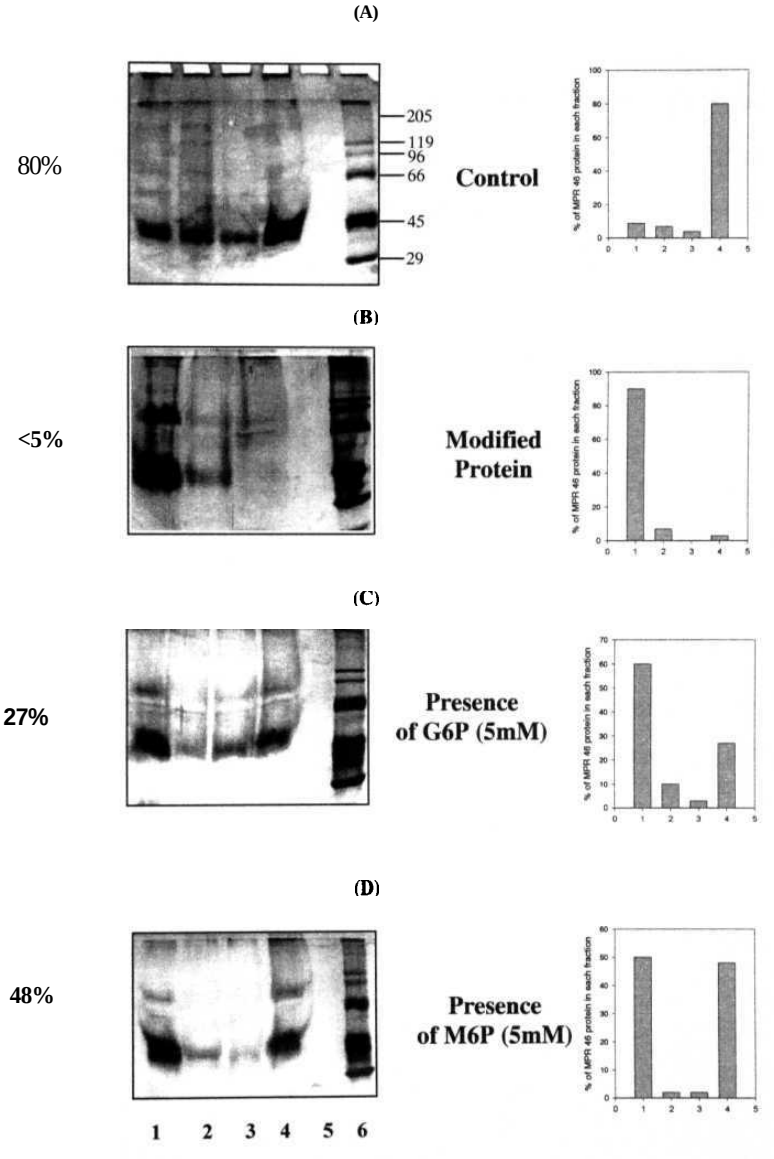

(A)
80%
205
119
96
66
45
29
Control
% of MPR 46 protein in each fraction
(B)
<5%
Modified Protein
% of MPR 46 protein in each fraction
(C)
27%
Presence of G6P (5mM)
% of MPR 46 protein in each fraction
(D)
48%
Presence of M6P (5mM)
% of MPR 46 protein in each fraction
1 2 3 4 5 6

Figure 16

Figure 17: Modification of histidine and arginine residues in the purified chicken MPR 46 protein. Modification of Histidine residue without (A) or with 5mM mannose 6-phospahte (Bj. Modification of Arginine residue without (C) or with 5mM mannose 6-phosophate (D).

Lanes I, unbound; 2, wash; 3, glucose 6-phosphate eluate; 4, mannose 6-phosphate eluate.

% mentioned on the left and right side of the gel pictures is donoted % of MPR 46 eluted fromthe PM gel with 5mM mannose 6-phosphate.

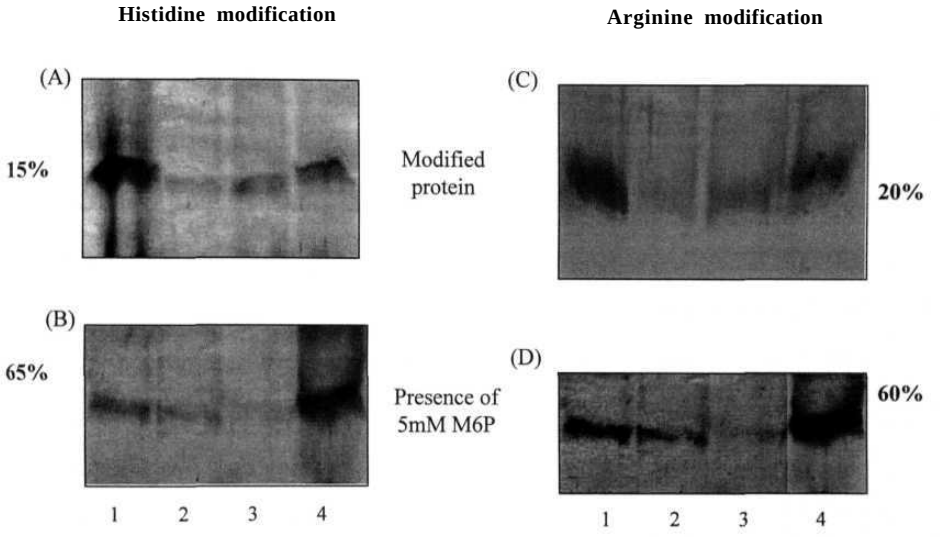

Histidine modification
Arginine modification
(A)
15%
(B)
65%
Modified protein
Presence of 5mM M6P
(C)
20%
(D)
60%
1 2 3 4
1 2 3 4

Figure 17

DISCUSSION:

Chemical modification studies of the purified proteins using group specific reagents have been extensively used as useful tools to identify the role of specific amino acids in defining the function of proteins. The role of specific amino acids conferring lectin activity in purified lectins has been well established using chemical modification studies (Radha and Siva Kumar, 2003).

In the present study, MPR 46 proteins from goat and chicken liver were purified to homogeneity and were used to identify the amino acids in the proteins that are essential for ligand binding. The unmodified purified receptors served as controls. As it is known that the human MPR 46 protein contains histidine and arginine residues that are critical for ligand binding, (for review Dahms and Hancock, 2002), we have employed chemical reagents that can specifically modify histidine and arginine residues in the purified receptors. The ability of the modified protein to bind on PM gel was determined. Protection experiments were also carried out using 5mM glucose 6-phosphate and 5mM mannose 6-phosphate in separate experiments to determine the specificity of the modification protocol.

From the results it is evident that the unmodified protein binds to PM gel to an extent of 80%. However, on modification of the histidine residues in goat MPR 46, the protein could be bound to an extent of only 8% on PM gel. When modification was carried out in the presence 5mM glucose 6-phosphate, the protein showed 14% binding on the gel. On the other hand, when modification was carried out in the presence of 5mM mannose 6-phosphate, the protein showed 68% binding on gel, suggesting in presence of the specific sugar mannose 6-phosphate (5mM) there was significant protection of modification.

Similar experiments were carried out with the goat MPR 46 protein where arginine residues were modified. There was <5% binding of the protein on PM gel. In the presence of 5mM glucose 6-phosphate, the binding was found to be 27% and in the presence of mannose 6-phosphate, the binding was found to be 48%.

For the purified chicken MPR 46 protein, modification of the histidine and arginine residues resulted in 15% and 20% binding on PM gel respectively. Protection experiments carried out in the presence of 5mM mannose 6-phosphate, revealed that the protein bound on the gel to the extent of 65% and 60% respectively.

The results obtained thus, suggest the possible involvement of arginine residues in both the proteins (goat and chicken MPR 46) for ligand binding.

For human MPR 46 as well as MPR 300 proteins, the involvement of specific arginine residues in ligand binding has been well-established (Dahms and Hancock, 2002). It is interesting to note from the studies carried out by other workers (Zhou *et a!.*, 1995; Udaya Lakshmi *et al.*, 2000) that the MPR 300 protein from different animal species also has specific arginine residues that are important for ligand binding, (The ligand binding region is highly conserved among different animal species) further suggesting on the importance of the structure and function of the two receptors. Further evidence for the possible involvement of arginine residues in the goat receptor is substantiated from molecular cloning studies, which has been described in Chapter V.

Since the full length clone for chicken MPR 46 is not available, from this study we can only conclude that arginine residues are important for ligand binding. In addition to this, the histidine modified sample also exhibited decreased binding on PM gel, compared to the control. However, the significance and importance of this finding needs to be established.

CHAPTER - IV

Interaction of purified goat and chicken MPR 300 proteins with human IGF-II

INTRODUCTION:

Among the two receptors characterized from mammals, only MPR 300 has so far been shown to be a multifunctional protein which in addition to binding mannose 6-phosphate containing lysosomal enzymes, also binds IGF-II and other ligands such as retinoic acid and thyroglobulin (Hille-Rehfeld, 1995; Kang *et al,* 1998). In humans it has been shown that this receptor plays an important role in controlling the extracellular level of the insulin-like growth factor II (IGF-II) by mediating its binding at the cell surface and delivery to lysosomes (Kornfeld 1992). The luminal ligand-binding domain of MPR 300 contains 15 internal repeats, which are homologous to the luminal domain of MPR 46. It is therefore of interest to study the evolution and functions of these receptor proteins in order to analyze their biochemical and ligand binding properties. Receptors purified from rat, bovine, human and some other mammalian species such as the opossum exhibited IGF-II binding abilities (Schmidt *et al,* 1995) and the IGF-II binding domain in mammals has been localized to repeat 11 of this multifunctional receptor. The biochemical and immunological properties of the purified MPR 300 protein from goat, chicken, garden lizard, fish and *Unio* resemble those of the mammalian receptor (Siva Kumar *et al,* 1997; 1999; Udaya Lakshmi *e al,* 1999,). Further a partial cDNA clone for the fish MPR 300 has been shown to contain the highly conserved mannose 6-phosphate binding region in the third domain similar to other vertebrate receptors (Udaya Lakshmi *et al.,* 2000). When purified MPR 300 from chicken and *Xenopus* (non-mammalian vertebrates) was tested for its binding to human IGF-II, the receptors failed to bind IGF-II, under the conditions used and it was suggested that possibly acquisition of IGF-II binding site by the receptor is a late event in evolution (Canfield and Kornfeld 1989; Clairmont and Czech 1989). In a

recent study it has been shown that the MPR 300 from the earliest non-mammalian vertebrate, fish binds human IGF-II under specific conditions (Mendez *et al,* 2001). This recent observation led us to believe that possibly the IGF-II binding property of the MPR 300 is also conserved in all non-mammalian vertebrates as our earlier studies demonstrated that the arginine residue in domain 3 of the MPR 300 that is responsible for ligand binding is evolutionarily conserved (Udaya Lakshmi *et al,* 2000). The objective of the present study was to investigate whether the MPR 300 receptors purified by us from goat and chicken possess IGF-II binding ability. To address this question four different assays were done. i) a cross-linking assay using ^{125}I-IGF-II ii) a ligand blot assay using biotinylated IGF-II. iii) a filter binding assay using ^{125}I-IGF-II and iv) an internalization assay employing CEF cells and ^{125}I-IGF-II as the CEF cells have been shown to contain MPR 300 protein (Matzner *et al.,* 1996).

MATERIALS:

Biotinylated IGF-II	:	Generously provided by Prof.Dr.Thomas Braulke, Hamburg, Germany.
BSA	:	Sigma
DMEM medium	:	Sigma
DSS (Disuccinimidyl suberate)	:	Sigma
ECL (Enhanced chemiluminiscence) reagent	:	Pierce
HEPES	:	Sigma
Nitocellulose filters	:	Millipore
Polyethylene glycol	:	Sigma
Tris	:	Qualigens
Triton X 100	:	Sigma
γ-globulin	:	Sigma

Reagents:

Krebs Ringer phosphate buffer:

(A) 0.15MNaCl

(B) 0.15M KCl

(C) 0.11M $CaCl_2$

(D) 0.15M $MgSO_4.7H_2O$

(E) 10mM Na_2HPO_4 pH 7.4

To prepare the Krebs Ringer phosphate buffer above solutions were mixed in the following proportions:

100 parts of A + 4 parts of B + 3 parts of C + 1 part of D + 20 parts of E.

Equipment

γ-ray counter	:	ECIL, India.

METHODS:

Affinity cross-linking of ^{125}I IGF-II with purified MPR 300 from goat and chicken

Crosslinking studies were carried out as described by Causin *et al.*, (1989). About 5 µg of purified receptors were acetone precipitated and the precipitate was solubulized in 37ul of 50mM-sodium phosphate, pH 7.4, containing 150 mM NaCl, 0.1% Triton X-100 and 0.1% bovine serum albumin and incubated for 20 min at 4°C with or without 200nM or 400nM unlabelled IGF-II. 13µl of ^{125}I-IGF-II, (2,50,000 cpm) was added and incubated for 3h at 4°C. Crosslinking of bound IGF II with receptor was performed with 1mM-DSS by incubating the reaction mixture for 15 min. at 4°C. The reaction was stopped with 5 µl of 1 M-Tris/HCl pH 7.4, 2.75 µl of 20% SDS and 3 u1 of glycerol (100%). The reaction mixture was heated at 95°C for 5 min. and the samples were subjected to 7.5% SDS-PAGE. The radiolabeled bands were visualized by autoradiography using a Kodak X-ray film with light intensifying screens.

Immunoprecipitation

Immunoprecipitation was carried out as described by Mendez *et al.*, (2001). About 6 µg of purified mannose 6-phosphate receptor proteins from both goat and chicken were taken and incubated for 16 h at 4°C with ^{125}I-IGF-II (25 pM) in HEPES buffer pH 7.6. After the incubation, cross-linking was carried out with 1mM DSS on ice for 15 min. The reaction was stopped by adding 10mM Tris-HCl buffer pH 7.4. Subsequently, receptor preparations were incubated with or without 10 µg of affinity purified IgG against the goat MPR 300 protein for 8h at 4°C with constant agitation. The immune complexes were then

collected using Protein A-agarose and washed with PBS containing 0.05% Tween 20. The complexes were solubulized by boiling at 95^0C for 5 min. in SDS sample buffer and subjected to 7.5% SDS-PAGE. The radiolabeled bands were visualized by autoradiography as described above.

Binding of biotinylated IGF-II to the purified MPR 300 from goat and chicken:

Binding was carried out as described by Shalamanova *et al.*, (2000). About 1 ug of purified goat and chicken liver receptor (MPR 300) were electrophoresed on 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred on to a nitrocellulose membrane overnight at 4°C and the membrane was blocked for one hour at room temperature in PBS containing 0.1% Tween 20 and 1% BSA and incubated overnight at 4°C in monobiotinylated-IGF-II (20ng/ml) in PBS containing 0.1% Tween 20 and 1% BSA. The membrane was washed consecutively with PBS containing 0.1% Tween 20 (1 x 15 min, and 4 x 5 min) and incubated with peroxidase-conjugated streptavidin for one hour at room temperature in PBS containing 0.1% Tween 20 and 1% BSA. Finally the membrane was washed consecutively with PBS, 0.1% Tween 20 (1 x 15 min, and 4 x 5 min). The membrane was treated with ECL reagent (Pierce) for one minute and the blot analyzed using a Kodak X-ray film.

Quantitation of ^{125}I-IGF-II binding to the purified MPR 300 from goat and chicken using the Polyethylene Glycol Precipitation Method

This was carried out as described previously (Clairmont and Czech 1989) with few modifications. 0.1ml of a stock solution of 4nM ^{125}I-IGF-II and unlabeled IGF-II at 0, 10, 40, 100 and 400 nM were combined with an equal volume of a solution of purified receptors (goat and chicken MPR 300) containing 4 ug of protein. Following incubation

for 2h at 3°C, 0.5ml of 0.9mg/ml bovine γ-globulins in 0.1M sodium phosphate buffer, pH 7.4, and 0.5% polyethylene glycol were added to each sample, the sample was mixed and incubated at 0°C for 15 min. This mixture was then filtered through nitrocellulose filters (0.45μm) which were blocked by incubation in Krebs Ringer phosphate buffer containing 1% bovine serum albumin. The filters were washed three times with 8% polyethylene glycol and the y-radiation from the filters measured in a y-ray counter.

Cell culture studies

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

IGF-II binding assays in CEF cells

Internalization assay was carried out as described recently for fish receptor (Mendez *et al.*, 2001). Cells grown in 3cm petriplates (2 million cells) as confluent monolayers were washed twice for over 2h in 0.1 M HEPES containing 0.12M NaCl, 5mM KC1, 1.2mM $MgSO_4$, 8mM glucose and 0.5% BSA (binding buffer, pH 7.6) and were incubated for 4h at 4°C in 0.5ml of the same buffer containing ^{125}I-IGF-II (25 pM) in the presence or absence of unlabelled IGF-II (200nM), insulin (100nM) , mannose 6-phosphate (5mM) and affinity purified IgG for MPR 300 protein (15 μg). Subsequently, the cells were washed twice with the binding buffer and burst open with 0.5N NaOH for the determination of radioactivity.

RESULTS:

Purification of the MPR 300 protein by affinity chromatography:

The total membrane protein extracts obtained from the liver tissues of mammalian (goat) and non-mammalian vertebrate (chicken) were passed through two separate PM gels, and the gels were washed extensively with the column buffer. The bound protein was eluted from both gels with 5mM mannose 6-phosphate in the column buffer. The eluted fractions were concentrated and aliquots subjected to SDS-PAGE under reducing conditions. Consistent with our earlier findings (Siva Kumar *et al.*, 1996) in both the species the purified MPR 300 behaved as a single protein band corresponding to molecular mass of about 300 kDa (Figure 18). The yield of the MPR 300 was comparable with earlier data.

Affinity crosslinking of ^{125}I- IGF-II to MPR 300 from goat and chicken

Purified receptors were subjected to affinity labeling using ^{125}I-IGF-II and disuccinimidyl suberate (DSS) as the cross-linker in the presence of 200nM and 400nM of unlabeled IGF-II. Purified goat and chicken MPR 300 protein showed efficient affinity labeling with ^{125}I-IGF-II under the conditions used, as is evident from the autoradiogram suggesting that both goat and chicken MPR 300 proteins bind human IGF-II. In the presence of unlabeled IGF-II (200nM) (Figure 19A), there was a partial inhibition of affinity labeling and in the presence of unlabeled IGF-II (400nM) (Figure 19B), there was almost complete inhibition of affinity labeling.

In a separate experiment the identity of this cross-linked product was further confirmed by immunoprecipitation using affinity purified goat MPR 300 IgG (Figure 19C). The results obtained clearly demonstrate that the cross-linked product seen in both species is the MPR 300 protein.

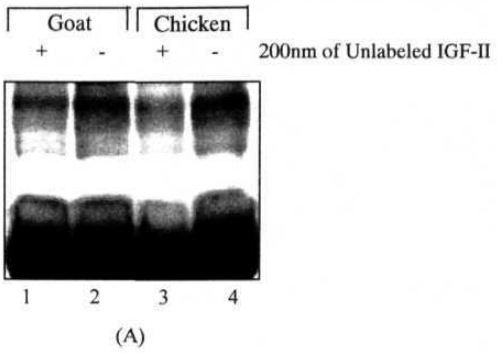
Figure 18: *10% SDS-PAGE of the purified Mannose 6-phosphate receptor proteins (MPR 300) under reducing conditions. Lane* ***1****, Purified goat receptor* ***(3μg)****, and Lane 2, purified chicken (2ug). Arrow indicates position of* ***MPR*** *300.*

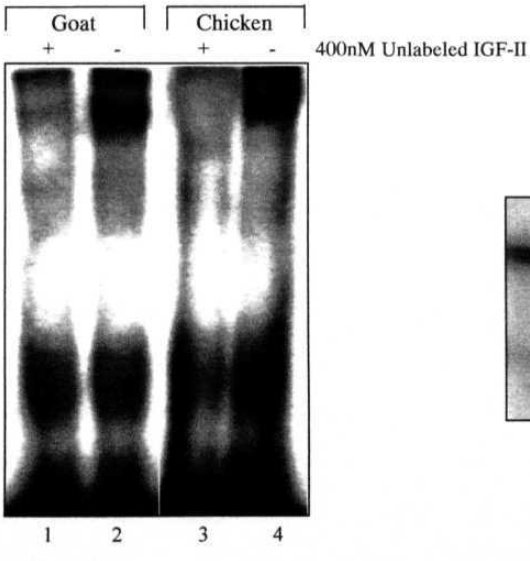
Figure 18

Figure 19: *(A). Affinity labeling of the purified receptors with* ^{125}***I IGF-II.*** *Lanes I, 2 goat, 3, 4 chicken. Lanes 1, 3, in presence of 200nm of unlabeled* ***IGF-II****, lanes 2 and 4 without unlabeled* ***IGF-II****.*

(B). *Affinity labeling of the purified receptors with* ^{125}***I IGF-II.*** *Lanes 1, 2 goat, 3, 4 chicken. Lanes 1, 3, in presence of 400nm of unlabeled IGF-II, lanes 2 and 4 without unlabeled IGF-II.*

(C). ***Immunoprecipitation*** *of* ***the IGF-II-MPR*** *300 cross-linked* ***product.*** *with affinity purified goat MI'R 300* ***IgG****. Lane 1, goat, Lane 2, chicken.*


Goat
Chicken
+ - + -
200nm of Unlabeled IGF-II
1 2 3 4
(A)


Goat
Chicken
+ - + -
400nM Unlabeled IGF-II
1 2 3 4
(B)

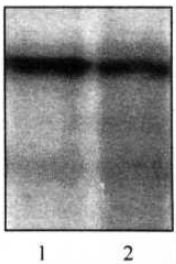

1 2
(C)

Figure 19

Binding of biotinylated IGF-II to purified MPR 300 from goat and chicken

The ability of the purified receptors from goat and chicken to bind monobiotinylated IGF-II was tested as described under methods. Both goat and chicken MPR 300 showed binding to IGF-II (Figure 20).

Affinity of ^{125}I-IGF-II to MPR 300 from goat and chicken:

To analyze the binding property of the purified receptors to ^{125}I-IGF-II, the affinity purified receptor (4 μg) was incubated with 2nM ^{125}I-IGF-II in a final volume of 200 μl in the presence of increasing concentrations of unlabelled IGF-II as described under methods. The radioactivity bound to the nitrocellulose filter was measured using a y-ray counter and the data was analyzed by Scatchard analysis (Figure 21). This suggests that both goat and chicken receptor show IGF-II binding.

Internalization assay of the ^{125}I-IGF-II:

In order to analyze if the Chicken Embryonic Fibroblast (CEF) MPR 300 protein can internalize ^{125}I-IGF-II, various experiments were carried out as described under methods. The amount of radioactivity internalized by the CEF cells when incubated with 25pM of ^{125}I-IGF-II was taken as 100% (control). The results are shown in Figure 22. When the cells were pre-incubated with either unlabeled IGF-II or affinity purified specific IgG for goat MPR 300 protein followed by incubation with ^{125}I-IGF-II, internalization was inhibited. When the cells were pre-incubated either with insulin (100nM) or 5mM mannose 6-phosphate followed by incubation with ^{125}I-IGF-II, internalization of the radioactivity could be seen. The data indicates that the MPR 300 from CEF cells has both mannose 6-phosphate as well as IGF-II binding sites and is able to internalize ^{125}I-IGF-II.

Figure 20: *Interaction of purified goat and* ***chicken*** *receptor with Biotinylated IGF-II. Details as given under text. Lane I goat and lane 2 chicken. Arrow indicates position of the receptor.*

Figure 21: *Scatchard analysis of IGF-II binding to affinity purified MPR 300 proteins from (a) goat and (b) chicken in presence of increasing concentrations of unlabeled IGF-II. Details as given in text. B, Bound IGF-II and B/F bound/free. Results are average of five and three experiments for goat and chicken respectively.*

Figure 22: *Internalization of labeled IGF-II by CEF cells. Internalization of labeled IGF-II assessed after preincubation of the cells with unlabeled IGF-II (A), affinity purified anti-goat* ***IgG*** *(B), insulin (C),* ***mannose*** *6-phosphate (D) and control (E).*

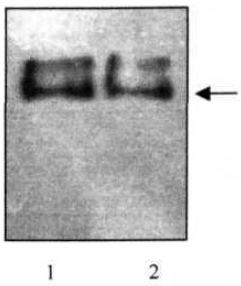


Figure 20

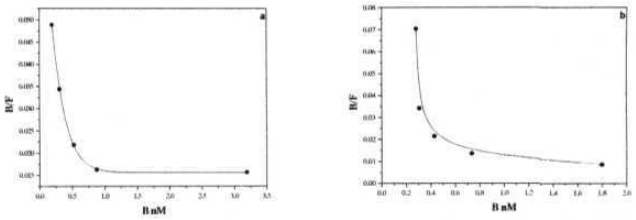


Figure 21

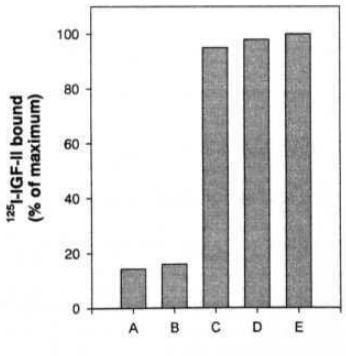


Figure 22

DISCUSSION:

Mannose 6-phosphate receptors, MPR 300 and MPR 46 have earlier been affinity purified employing phosphomannan Sepharose gel from different mammals and non-mammalian vertebrates (Siva Kumar *et al.,* 1997; 1999) and these proteins exhibited similar biochemical and immunological properties as that of the well studied mammalian receptors (Hille-Rehfeld, 1995). The luminal domain of the MPR 46 displays 14-37% similarity to the 15 internal cassettes of MPR 300 as shown for the bovine MPRs (Lobel *et al,* 1998). This finding led to the assumption that MPR 300 has evolved from MPR 46 by repeated gene duplication events and raised the question at what stage in evolution MPR 300 has occurred for the first time. First evidence for the presence of both MPR proteins among the CEF cells came from the studies of Matzner *et al.,* (1996).

Earlier studies have shown that different mammalian MPR 300 proteins bind human IGF-II, (Schmidt *et al.,* 1995). Though it has been shown that the chicken and *Xenopus* MPR 300 lack the IGF-II binding site under the conditions used by the authors (Canfield and Kornfeld 1989; Clairmont and Czech 1989), in a recent study it has been well established that the fish MPR 300 has the IGF-II binding ability (Mendez *et al.,* 2001) and in this study the authors suggested that failure to detect IGF-II binding in chicken and *Xenopus* by earlier authors is possibly due to the different conditions that were employed in their study. In the light of this recent finding that fish receptor binds IGF-II (Mendez *et al.,* 2001), in the present study we have carried out detailed analysis on the ability of purified goat MPR 300 (which has not been studied earlier) as well as that of chicken MPR 300 protein to bind human IGF-II.

In order to conclusively establish that the IGF-II binds to purified MPR 300, we have made cross-linking studies where radioiodinated IGF-II was cross-linked with the purified receptors employing 1mM DSS. The results indicate that the goat and the chicken receptors showed affinity cross-linking with labeled IGF-II. When these experiments were conducted in presence of 200nM unlabeled IGF-II, inhibition of affinity labeling was observed in both species (Figure 19A, lane land 3) suggesting that the interaction seen with goat and chicken receptors and labeled IGF-II is indeed specific. When the concentration of the unlabeled IGF-II was increased to 400nM, using goat receptor alone there was complete inhibition of affinity labeling (Figure 19B, lane 1 and 3). Similar observations were noticed with the purified rat and human MPR 300 protein (Clairmont and Czech 1989; Causin *et al.,* 1989).

Further confirmatory evidence that the cross-linked product represents the MPR 300 and the labeled IGF-II in both species, was established by specific immunoprecipitation of the product with affinity purified goat MPR 300 IgG (Figure 19C). It has been shown that the receptors from different vertebrate species can be recognized by goat MPR 300 antiserum (Siva Kumar *et al,* 1997). Further, the fish MPR 300 protein has been shown to react specifically with anti-MPR 300 rat antiserum (Mendez *et al.,* 2001).

In recent years biotinylated IGF-IIs have been employed for determining their binding abilities to their receptors in a dot blot analysis using simple and sensitive detection by streptavidin-coupled HRP and enhanced chemiluminiscence (ECL). The high reliability and sensitivity of the biotinylated IGF-II was exploited in this study to investigate the binding of goat and chicken MPR 300 to IGF-II. The efficient binding of both receptors to

biotinylated IGF-II in this assay further support the above experimental findings that the MPR 300 from both species indeed binds human IGF-II.

The results of the filter-binding assay using purified goat and chicken MPR 300 proteins suggest that both receptors bind human IGF-II under the conditions used. Scatchard analysis indicates a Kd value of 12.5nM for goat MPR 300 and 11.1nM for the chicken MPR 300. The Kd values of bovine and opossum were reported to be 0.2nM and 14.5nM respectively (Dahms *et al.,* 1993) and that of fish was found to be 0.12nM (Mendez *et al,* 2001). The results obtained for goat receptor is consistent with the findings of other workers who showed that mammalian MPR 300 has IGF-II binding ability (Schmidt *et al,* 1995). The only variance is with respect to the chicken receptor.

In order to support the data obtained for chicken receptor, we employed CEF cells in our studies, as these cells have been already shown to contain the MPR 300 protein (Matzner *et al,* 1996) and there is no published information on the use of these cells to analyze the IGF-II binding and internalization.

Experimental results indicate that the internalization of the labeled IGF-II by the CEF cells is mediated by the MPR 300 protein. When the cells were preincubated with cold (unlabeled) IGF-II (200nm or 400nm) followed by labeled IGF-II, 86% inhibition of internalization was observed. When cells were incubated with affinity purified MPR 300 IgG (15 μg), followed by labeled IGF-II 84%, inhibition of internalization was observed. However, when cells were incubated with insulin (100nM), only 5% inhibition could be seen. When the binding sugar, 5mM mannose 6-phosphate was used to preincubate the cells, followed by labeled IGF-II only 2% inhibition was seen. It is already well

established that the mannose 6-phosphate binding site and the IGF-II binding site in mammalian MPR 300 are distinct (Schmidt *et al,* 1995) and hence no effect of internalization in presence of mannose 6-phosphate is seen. Similarly insulin which has no binding to the receptor also shows no specific effect. However, cold IGF-II either at 200nm or 40()nm concentration showed distinct inhibitory effect. Additionally, an affinity purified goat MPR 300 antibody that is known to recognize mammalian and chicken MPR 300 protein (Siva Kumar *et al,* 1999) shows significant inhibition. Taken together, these results support the involvement of MPR 300 in internalization of ^{125}I-IGF-II in CEF cells.

In mammals, the IGF-II binding site of MPR 300 has been localized to amino acid residues from 1508-1566 in the amino terminal domain 11 (Schmidt *et al,* 1995). cDNA sequence obtained for chicken MPR 300 (Zhou *et al,* 1995) from domain 11 reveals that there is divergence in the amino acid sequence compared to human MPR 300. However, in the present study we found that the CEF cells which are known to contain MPR 300 protein, can internalize labeled IGF-II and the internalization is blocked by MPR 300 specific IgG but not by mannose 6-phosphate. These data suggest that possibly the CEF MPR 300 internalizes the labeled IGF-II. In view of the recent observation that the fish MPR 300 can bind IGF-II, the results obtained by us additionally confirms that the MPR 300 protein among the non-mammalian vertebrates also has the ability to bind IGF-II. Due to the non-availability of a reptilian cell line and a purified receptor from that species this was not tested.

Since, the complete fish MPR 300 sequence is not published and the evidence that chicken MPR 300 is able to bind labeled IGF-II, (though primary sequence suggests a change in the amino acid that is critical for binding IGF-II in chicken MPR 300), it is possible that

other regions of the receptor may be aiding in the binding of IGF-II. This can possibly be validated as in a recent study it has been shown that the IGF-II binding site of the mammalian IGF-II/MPR is bipartite: the primary determinants for binding reside in domain 11 while sequence elements within domain 13 contribute a ~5-10 fold enhancement of the binding affinity of the receptor for IGF-II (Devi *et al,* 1998; Grimme *et al,* 2000; Linnell *et al,* 2001).

Four lines of evidence suggest that the IGF-II binding property is exhibited by both the purified receptors. First, purified receptors show affinity cross-linking with labeled IGF-II and this is specific as evidenced by immunoprecipitation of these employing goat MPR 300 specific IgG that is known to immunoprecipitate goat, as well as, chicken MPR 300 proteins. Second, both the receptors show specific reactivity with biotinylated IGF-II. Third, both the receptors (goat and chicken MPR 300) bind radiolabeled IGF-II in a filter-binding assay. Fourth, the CEF cells show specific internalization of the labeled IGF-II which can be inhibited by unlabeled IGF-II and affinity purified goat MPR 300 IgG but not by insulin or mannose 6-phosphate. The fact that the goat receptor binds to phosphomannan Sepharose gel in a mannose 6-phosphate dependent manner and its ability to bind human IGF-II suggests that it possibly functions in a similar way as the human or rat liver MPR 300 protein. These observations are consistent with the recent finding on the imprinting of m6p/IGF2 receptor gene in mammals (Killian *et al.,* 2000). The goat MPR 300 has been shown to contain repeating cassette structures in the extracellular domain similar to other known receptors (Udaya Lakshimi *et al,* 2000) discussed in chapter V. It remains to be established whether the goat receptor has any additional ligand binding

properties like the other mammalian receptors. Table 7 below summarizes the information on the IGF-II binding ability of known MPR 300 proteins.

Animal Species	IGF-II binding property	Reference
MAMMALIAN		
Human	Yes	Tong *et al.*, 1988.
Bovine	Yes	Morgan *et al.*, 1987.
Rat	Yes	
Goat	**Yes**	**Present study**
Opossum	Yes	Dahms *et al.*, 1993.
Kangaroo	Yes	Yandell *et al.*, 1999.
NON-MAMMALIAN VERTEBRATES		
Chicken and Frog	No	Canfield and Kornfeld., 1989; Clairmont and Czech, 1989.
Chicken	**Yes**	**Present study**
Reptilian	ND	
Fish	Yes	Mendez *et al., 2001.*
INVERTEBRATES		
Mollusc	ND	

ND: Not Determined

***Table7**: IGF-II binding ability of some of the known MPR 300 proteins.*

CHAPTER - V

Molecular cloning of goat MPR proteins

INTRODUCTION:

The existence of two homologous mannose 6-phosphate receptors with overlapping but distinct functions has raised a question at what stage in the phylogenetic tree the two receptors have occurred for the first time. The cDNA sequences of mammalian and chicken MPR 300 proteins have revealed that they contain three distinct domains, the extracytoplasmic (or luminal), the transmembrane and the cytoplasmic domains. The extracytoplasmic domain is comprised of 15 repetitive units which share significant sequence similarity with each other and also with the single unit that constitutes the extracytoplasmic domain of MPR 46.

The goat receptor proteins have been purified and characterized biochemically and immunologically in the present study. To understand their structure-function relationships, we carried out experiments to obtain a partial cDNA clone for the goat MPR 300 and a full length cDNA clone for the goat MPR 46. Since the sequences of some mammalian species are already known, the strategy adopted was to design primers based on the multiple sequence alignment of known MPR proteins. These primers were used in RT-PCR experiments with goat liver RNA to amplify cDNA fragments which were then cloned and sequenced.

MATERIALS

Instruments:

Thermocycler Gene Amp PCR 2400 machine	Stratagene, Eppendorf
Transilluminator Model IL-400-M	Bachofer, Reutlingen
373 A DNA sequencing system	Applied Biosystems

Chemicals:

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

Kits used for molecular biological work:

▪ QIAgen RNeasy total RNA isolation kit	Diagen,Hilden
• First strand cDNA synthesis kit (QIAgen, Omniscript)	Diagen,Hilden
• HotStar *Taq* polymerase PCR QIAgen kit	Diagen, Hilden
• HotStar *Taq* polymerase Master Mix QIAgen kit	Diagen, Hilden
• QIAquick gel extraction kit	Diagen, Hilden
• TA cloning kit	Invitrogen, Groningen and MBI Fermentas
• QIAgen plasmid mini kit	Diagen, Hilden

• Dye terminator cycle sequencing kit	Applied **Biosystems**
• Random primer DNA labeling kit	MBI Fermentas
• Ultra pure dNTP set	MBI Fermentas

Enzymes for molecular biological work:

Restriction enzymes	New England Biolabs and MBI fermentas

Plasm id ***DNA vectors:***

pCR 2.1 TA cloning vector (Invitrogen)

pTZ57R TA cloning vector (MBI Fermentas)

DNA standards:

DNA-Ladder Invitrogen, MBI fermentas

Reagents used for molecular biology work:

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

50x TAE *2* M Tris-Base 0.1 M EDTA, pH adjusted to 8.0 with acetic acid.	***TE*** 10 mM Tris-HCl pH 7.5 1 mM EDTA.
50 x Denhardt Solution 5 g Ficoll 5 g Polyvinylpyrrolidine 5 g BSA The volume was adjusted to 500 ml with deionized water.	***LB medium*** 10 g of NaCl 10 g of Tryptone 5 g of Yeast extract, pH adjusted to 7.0 with 5 N NaOH. The final volume was made up to 1 liter with deionized water, autoclaved and used.
LB agar (per liter) 10 g of NaCl 10 g of Tryptone 5 g of Yeast extract 20 g of agar The pH was adjusted to 7.0 with 5 N NaOH, the final volume was made up to 1 liter with deionized water, autoclaved and poured into petri dishes (25 ml/10 cm plate or 60 ml/ 14.5 cm plate).	***20x SSC Buffer*** 175.3 g of NaCl 88.2 g of Sodium citrate 800 ml of deionized H_2O. The pH was adjusted to 7.0 with a few drops of 10 N NaOH, deionized H_2O was added to make the final volume to 1 liter.
Preparation of Antibiotics Ampicillin/Kanamycin:25 mg/ ml stock solution of the sodium salt of Ampicillin/Kanamycin in water was prepared. The pH adjusted to 8.0 with 2 N NaOH. It was sterile filtered and stored in aliquots at -20°C.	***Preparation of LB-Agar*** ***Plates with Appropriate Antibiotic*** LB-Agar was autoclaved and cooled to 55°C. Ampicillin/Kanamycin was added to a concentration of 50 µg / ml and poured into petri plates.
Denaturation Buffer 1.5 M NaCl, 0.5 M NaOH 81.6 g NaCl and 20 g of NaOH was dissolved in deionized water and the final volume was made up to 1 liter.	***Neutralization Buffer*** 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA 10x Tris-buffer (100 ml) was prepared, NaCl and EDTA (pH 8.0) were added to a final concentration of 1.5 M and 1 mM respectively. The volume was adjusted to 1 liter.

Prehybridization *Buffer* 48% Formamide 4.8x SSC 10 mM Tris-HCl pH 7.4 1%SDS 1% Denhardt solution 10% Dextran sulfate. 100 μg/ ml Salmon sperm DNA. Stored at 4°C.	*3 M Sodium acetate* 408.1 g sodium acetate (tri hydrated) was dissolved in 800 ml water, the pH was adjusted to 5.2 with acetic acid. The volume was made up to 1 liter, autoclaved and used.
Denatured Salmon Sperm DNA Salmon sperm DNA (sodium salt) was dissolved in water at a concentration of 10 mg / ml. The solution was stirred on a magnetic stirrer for 2-4 hours at room temperature to dissolve DNA. The DNA was passed through a 20-G gauze needle, boiled for 10 min. (at 100°C), sonicated for 1-2 minutes and stored at -20°C in small aliquots.	20% *SDS* 20 g of Sodium dodecyl sulfate was dissolved in 100 ml water at 65°C and sterile filtered.
10x*MOPS Buffer* 20.93 g of MOPS (200 mM) 2.05 g of sodium acetate (50 mM) 1.86 g EDTA (10 mM) Dissolved in 300 ml DEPC-H_2O, pH was adjusted to 7.0 with 2N NaOH in DEPC-H_2O (for 500 ml, 15-16 ml of NaOH is required). After each step of addition of the base, the pH was controlled by taking an aliquot in an Eppendorf tube. The final volume was made up to 500 ml with DEPC-water.	***5x Loading Buffer*** 16 μl Saturated bromophenol blue 80 μl 500 mM EDTA, pH 8.0 720 μl 37% (=12.3 M) formaldehyde 2 ml 100% glycerol 3.084 ml Formamide 4 ml 10x MOPS buffer RNase-free water was added to 10 ml.
SOC medium (pH 7.0) 2.0 g Bacto-Tryptone 0.5 g Bacto-Yeast Extract 1 ml of sterile filtered 2 M Mg^{2+} stock (1 M $MgCl_2.6H_2O$/ 1 M $MgSO_4.7H_2O$), 1 ml of sterile filtered 2M glucose. Tryptone, yeast extract, NaCl and KC1 were added to 97 ml of deionized water, stirred to dissolve the contents. Then it was autoclaved and cooled to room temperature. Mg^{2+} and glucose were added to a final concentration of 20 mM each. The volume was made upto 100 ml with sterile deionized water and filtered through a 0.2 urn filter unit	***IPTG*** *(isopropylthio fi-galactoside) stock solution* ***(0.1 M)*** 240 mg of IPTG was dissolved in 10 ml of deionized water, sterile filtered and stored at 4°C. ***X-Gal*** 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside was dissolved in N,N'-dimethyl formamide, tubes covered with aluminum foil and stored at -20°C

METHODS:

Total RNA isolation using RNeasy QIAgen kit:

Important points to be considered while handling RNA:

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

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

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

Total RNA isolation from goat liver tissue:

- Liver tissue (20-50mg) was sliced using a sterile scalpel. Tissue was lysed in 0.6 ml lysis buffer [Buffer RLT containing highly denaturing GITC (guanidinium isothiocyanate) which immediately inactivates RNase to ensure isolation of intact RNA] containing 6 µl of p-M.E that was added to the buffer before use.

- **Homogenization:** A homogenous suspension of the tissue was made using a fine sterile eppendorf pistle and passing the suspension 5 times through a 20-G (ϕ 0.9 mm) needle fitted to a syringe. It was centrifuged for 3 min. and the clear supernatant was transferred to another sterile eppendorf tube.
- 600 µl (1 volume) of 70% ethanol was added (to provide appropriate binding conditions) to the homogenized lysate and mixed well by pipetting up and down (A precipitate may form after the addition of ethanol).
- 700 µl of sample was applied on a RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at 13,000 rpm (Eppendorf centrifuge).
- The above step was repeated for rest of the sample.
- The column was washed with 700 µl of wash buffer RWl by applying the buffer on the RNeasy spin column and centrifuging for 15 seconds at 13,000 rpm.
- The flow through and collection tube were discarded.
- The RNeasy spin column was transferred to a new 2 ml collection tube and washed as above with 500 µl of buffer RPE.
- The column was re-washed with 500 µl of buffer RPE, centrifuged for 2 min. at 13,000 rpm to dry the RNeasy membrane (*It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent elution).
- The column was transferred into a new sterile 1.5 ml collection tube (supplied). 50 µl of RNase free water was pipetted directly onto the membrane, allowed to stand for one min. and centrifuged for 1 min. at 10,000 rpm to elute RNA.

Quantitation of nucleic acids (RNA/DNA):

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

The ratio between the absorbency readings at 260 nm and 280 nm gives an estimate of purity. Pure RNA/DNA preparation will have an A_{260}/A_{280} ratio of 2.0.

Spectrophotometric conversions

1A_{260} unit of dsDNA	=	50 µg/ml	= 0.15mM (in nucleotides)
1A_{260} unit of ssDNA	=	33 µg/ml	= 0.1mM (in nucleotides)
1 A_{260} unit of ssRNA	=	40 µg/ml	= 0.12mM (in nucleotides)
1mM (in nucleotides) of dsDNA	=	6.7A_{260} units	
1mM (in nucleotides) of ssDNA	=	10.0A_{260} units	
1mM (in nucleotides) of ssRNA	=	8.3A_{260} units	
The average MW of a deoxyribonucleotide base		= 333 daltons	
The average MW of a ribonucleotide base		340 daltons	

Denaturing agarose gel electrophoresis for RNA:

Components	% of Agarose gel		
	1	1.5	2
Agarose	0.5g	0.75g	1g
10x MOPS-buffer	5ml	5ml	5ml
DEPC-water	45ml	45ml	45ml

Table 8: Components for agarose gel preparation.

These components were heated in a microwave oven as per requirement, cooled to 65°C, 1-2 µl of EtBr (10 mg/ ml), 0.825 ml of formaldehyde (37%) were added, mixed well poured into a sealed electrophoresis boat trough fitted with a comb. The solidified gel was placed in the electrophoresis chamber.

Sample Preparation and gel run:

RNA sample (1-5 µg) was mixed with loading buffer (1 vol. of sample buffer to 4 vol. of RNA sample), cooked at 65°C for 5 min, chilled on ice and applied on the gel. The gel was run at 70 V for 1-2 hours.

Agarose gel electrophoresis for DNA:

DNA fragments were separated by agarose gel electrophoresis. Based on the size of DNA fragments to be resolved, the concentration of the gel varied as shown in Table 9.

Agarose gel %	Optimum resolution (bp)
0.5	1000-30000
0.7	800-12000
1.0	500-10000
1.2	400-7000
1.4	200-4000
2.0	50-2000

50-2000

Table 9: Percentage of agarose gel to be used for the separation of DNA fragments of different lengths.

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

Depending on the percentage of the gel, agarose was weighed and added to 35 ml of TAE, cooked in a microwave oven and cooled to 55°C. EtBr (10 mg/ ml) at a final concentration of 0.5 µg/ ml was mixed in it, poured into the gel trough fitted with combs and was allowed to cool to room temperature. The sample was mixed with sample buffer (10-20% (v/v)) and loaded in the wells. The gel was run for 1 to 2 hours in 1 x TAE buffer at 70V. The bands were visualized under UV.

Gel documentation:

Nucleic acids intercalated with the fluorescent dye ethidium bromide were visualized under UV light using transilluminator.

***Primer** designing:*

The parameters to be considered during primer selection are:

- **Length:** 18-30 nucleotides (Recommendable size of the primer is 24 nucleotides)
- **G/C Content:** 40-60%
- **T_m: T_m=** 2°C x (A+T) + 4°C x (G+C). Primer pairs were designed with a difference of + 2°C Tm values. Optimal annealing temperature was calculated as 5^0C below the estimated melting temperature.

- Complementarity of two or three bases at the 3'ends of primer pairs was avoided to reduce primer dimer formation.
- A complementary sequence within a primer sequence was avoided to reduce hairpin formation.
- A primer with A or T at 3' end was avoided, as it has greater tolerance of mismatch. It is always advantageous to have G/C at the 3' end.
- The computer program "Oligo analysis" or "Primer 3 (online)" was used for primer designing.

  Once primers were selected, a BLAST search was done to check for percentage homology with known sequences.

First strand cDNA synthesis (RT reaction) with QIAgen kit (Omniscript):

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

***First-Strand** cDNA Synthesis:*

- 1 -2 µg (in 5 µl) of the total RNA isolated was taken in a sterile eppendorf tube, denatured at 65°C for 5 min. and immediately chilled on ice.
- Following components were added to this according to the manufacturer's instructions (see Table 10).

Component	Volume (20 µl)/reaction	Final Concentration
10X Buffer RT	2.0 µl	1X
dNTP Mix (5mM each dNTP)	2.0 µl	0.5mM of each dNTP
Omniscript Reverse Transcriptase	1.0 µl	4 units (per 20 µl reaction)
Appropriate reverse primer	2 µl	20pmoles
Rnase-free water	Variable	
Template RNA	Variable	Upto 2 µg (per 20µl reaction)

Table 10: Composition of 1st Strand Synthesis Assay

- The above reaction mix was incubated at 37°C for 1 hour.
- The completed first strand reaction product was heated at 95°C for 5 min. (to denature RNA-cDNA duplex and to inactivate the reverse transcriptase) and chilled on ice. The denatured RT product was used for PCR amplification using specific sense and anti-sense primers.

PCR amplification (QIAGEN HotStar Taq DNA polymerase kit):

*HotStar **Taq** DNA Polymerase*

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

PCR Reaction:

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

Composition of PCR Amplification Assay:

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

The following were used for one PCR reaction for 20 µl reaction volume:

10 x PCR buffer	2 µl.
dNTP Mix	0.4 µ.l
Forward primer (sense)	1.2 µl.
Reverse primer (anti-sense)	1.2 µl.
Taq DNA polymerase	0.2 µl.
Template DNA (First strand cDNA (1-2 µl) or DNA)	x vol.

The total volume was made up to 20 µl.

Or

HostStar Master Mix 2X reaction mixture (QlAgen Master Mix Kit) was added in the following sequence for 20 µl reaction volume:

HostStar Master Mix reaction mixture	10 µl
Forward primer	1.2 µl
Reverse primer	1.2 µl
Template DNA (First strand cDNA (1-2 µl) or DNA)	x vol.

The total volume was made up to 20 µl.

Template DNA was added at the end. The PCR reaction was carried out using the thermal cycler program shown in the Table 11. The annealing temperature and other parameters were modified depending on the T_m and specificity of the primers used for amplification.

Reaction Step	Time (min.)	Temperature (°C)
Initial activation step	15	95
Repeated Number of Cycles 30-35		
Denaturation	1	94
Annealing	1	55
Extension	1	72
Final extension	10	72
Hold	α	4

Table 11: Thermal Cycler Program

Gel purification of PCR product (QIAEX II Gel extraction kit):

A preparative agarose gel was run, the cDNA band of expected length was cut with a clean sharp scalpel. The gel slice was weighed, 3 volumes of buffer QX 1 (solubilization and binding buffer) was added to one volume of gel (100 mg = 100 µl). QIAEX II was resuspended by vortexing for 30 sec. 30 µl of QIAEX II was added to the sample for 2-10 µl DNA or 10 µl of QIAEX II was added to the sample for <=2 µl of DNA. This was incubated at 50°C for 10 min. (or until the gel slice was completely dissolved). During incubation, the tube was agitated every 2 min. to make sure that the gel was dissolved completely (the color of the mixture should be yellow). If the colour of the mixture was orange or purple, 10 µl of 3M sodium acetate, pH 5.0 was added

and mixed until the colour turns yellow. The incubation was continued for an additional 5 min. The absorption of DNA to QIAEX II particles is only efficient at pH<= 7.5. Buffer QX1 contains a pH indicator which is yellow at pH <=7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH of DNA binding.

The mixture was centrifuged for 30 sec. in a tabletop centrifuge and supernatant was carefully removed using a pipette. The pellet was washed with 500 µl of buffer QX1, which removes residual agarose contaminants. The supernatant was removed by high speed centrifugation for 30 sec. The pellet was washed twice with 500 µl of buffer PE (wash buffer). After high speed centrifugation, the supernatant was removed carefully with a pipette. These washing steps remove residual salt contaminants.

The pellet was air-dried for 10-15 min or until it becomes white. (Do not vacuum dry, as this may cause overdrying and decrease elution efficiency).

Finally, DNA was eluted from the OIAEX II by the addition of 20 µl double distilled water and the pellet was resuspended by vortexing. This was incubated at room temperature for 5 min. to elute <=4 kb DNA or 5 min. at 50°C to elute 4-10kb DNA or 10 min. at 50°C to elute >10kb DNA.

TA Cloning (TOPO TA Cloning kit, Invitrogen):

Introduction:

Taq Polymerase has a non template-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The kit provides a quick, one step cloning strategy for the

direct insertion of a polymerase chain reaction (PCR) product into a plasmid vector (Figure 23B). The advantage of the kit is that one can eliminate any enzymatic modifications of the PCR product.

***Ligation (Quick** Ligation):*

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

- Ligation reaction was set up as follows

Fresh gel purified PCR product	x μl
Salt solution	0.5 μl
TOPO vector	1.0 μl

The final volume was made up to 5 μl with sterile water

- The ligation reaction was carried out at room temperature for 5min.
- Note: The ligated vector can be used for transformation or can be stored at-20^0C until ready for transformation.

Transformation into One Shot chemically competent cells (TOPO 10F'):

Before Start:

- A water bath was maintained at 42°C.
- A vial of SOC medium was thawed and incubated at 37°C.
- LB agar plates (two plates for each ligation/transformation) were equilibrated at 37^0C for a minimum of 30 minutes. Each plate was coated with kanamycin 50ug/ml final concentration, 40 μl of 40 mg/ ml X-Gal (chromogenic substrate for (3-galactosidase) 60 μl of 100 mM IPTG (isopropylthiogalactoside-used to maximize

the expression of genes cloned in expression vectors). The liquids were allowed to soak into the plates by incubating at 37°C for a minimum of 30 min.

Procedure for Transformation:

The vials containing the ligation reactions were centrifuged briefly to bring the contents down and then placed on ice.

3-5 µl of the ligated product was added into a vial of One Shot Chemically Competent *E.Coli* (TOPO 10F') cells and mixed gently (do not mix by pipetting up and down). The vial was incubated on ice for 30 min. A heat shock was given for 30 seconds in a 42^0C water bath. The vial was immediately removed from the water bath and placed on ice for 2-3 min. To this, 250 µl of SOC medium at room temperature was added. The vial was kept horizontal and shaken at 37°C for minimum 1 hour at 225 rpm in an orbital shaker incubator. 100 µl and 150 µl of the contents from the transformation vial was spread on separately labeled LB agar plates containing 50 µg /ml kanamycin, preabsorbed with X-gal and IPTG. The liquid was allowed to be absorbed into the agar plates and then inverted and placed in a 37°C incubator overnight. The transformed cells appear white in contrast to the untransformed blue colonies.

Plasmid DNA isolation (QIAprep plasmid DNA Isolation kit):

A single bacterial colony (white) was picked up with a sterile tip and inoculated into 5 ml medium (LB medium containing 50 µg/ ml Kanamycin). From an overnight culture plasmid DNA was prepared and digested with restriction enzymes. 5 ml of the overnight culture was taken into a sterile microcentrifuge tube and centrifuged at 10,000 rpm for 2 min. The supernatant was discarded and the pellet was resuspended in 250 µl of buffer P1 (suspension buffer). 250 µl of buffer P2 (lysis buffer) was added

and the tube was gently inverted 4-6 times (solution becomes viscous and slightly clear). The lysis reaction should not be allowed to proceed for more than 5min. 350 µl of buffer N3 (Neutralization buffer) was added and the tube was inverted immediately but gently 4-6 times to avoid localized precipitation (solution becomes cloudy) and then centrifuged at 13,000 rpm for 10 min. Meanwhile, QIAprep spin column was placed in a 2 ml collection tube.

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

Digesting plasmid DNA with restriction endonucleases:

About 1.0 to 2.0 µg of plasmid DNA was used for restriction analysis with the restriction enzyme as specified. To the plasmid DNA sample, 1 µl of enzyme (~10 Units/ µl) and 2 µl of 10X buffer were added (volume of the reaction mixture was made up to 20 µl with sterile water). Each restriction enzyme has a set of optimal reaction buffer conditions which are supplied by the manufacturer. The sample was incubated at 37°C for 1-2 hours. The product was analyzed by analytical agarose gel electrophoresis.

2', 3' Dideoxy NTP Dye Terminator Cycle Sequencing *(Applied Biosystem DNA Sequencing kit):* Sanger and Coulson (1977).

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

Primers: Vector specific sequencing primers M13 forward, M13 reverse (5pmoles/μl) were used. 100ng/ μl of plasmid DNA was used for each reaction.

The following reaction was set up

DNA	**Premix**	Primer
200-300ng	4 μl	10 pmoles

The volume was made upto 20 μl with sterile water.

Thermo Cycler Program:

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

Total number of cycles 25.

After PCR the sample was processed following standard conditions and subjected to sequence analysis on an automated DNA sequencer.

Random Primer Labeling: (HexaLabel *DNA Labeling Kit* ***MBI*** *Fermentas)*

The following components were added into a 1.5ml microcentrifuge tube:

DNA template (100ng)(MPR300/46)	10 μl
Hexanucleotide in 5X reaction buffer	10 μl
Deionized water	To make the volume up to 40 μl

The tube was vortexed for a few seconds and centrifuged in a microcentrifuge for 3-5 sec. The tube was incubated in a boiling water bath for 5-10min. and cooled in ice and centrifuged immediately for a brief period. To this following components were added sequentially:

dNTP mix (contains dATP, dGTP, dTTP) minus dCTP	3 µl
^{32}P dCTP (50uCi)	6 µl
Klenow fragment, exo- (5u)	1 µl

The tube was shaken, centrifuged in a micro-centrifuge for 3-5 sec. and incubated for 10 min. at 37"C. The labeling reaction was slopped by the addition of 1 µl of 0.5M EDTA, pH 8.0.

Determination of the % of label Incorporation

The percentage of incorporation was determined by DE-81 filter-binding assay.

1 µl of the reaction mixture was diluted to 1:100 with water. 5µl of this was spotted on two Whatmann DE-81 filters (1.5 x 1.5cm). The filters were dried under a heat lamp, one was kept aside and used directly for the determination of total dpm in the sample. The other filter was washed 3 times for 5min. in 10ml 7.5% (w/v) $Na_2HPO_4.12H_2O$ for the removal of the unincorporated dNTPs, followed by water and acetone. The washed filter was dried under a heat lamp. The unwashed and washed filters were counted in an appropriate radioactivity counter. The percentage of label incorporation into DNA is calculated as shown below:

$$\frac{\text{incorporated radioactivity (washed filter)}}{\text{total radioactivity (unwashed buffer)}} \times 100\%$$

The % incorporation was about 70% for the probes used.

Northern blot analysis (RNA Transfer):

Note: Before assembling the gel for transfer, the size of the gel was measured with a scale by exposing the gel briefly to UV at higher wavelength (312nm).

The transfer of total RNA from agarose gel to hybond-N nylon membrane was done using Pharmacia vacuum transfer unit. The gel was placed above the hybond-N nylon membrane and vacuum was applied. The gel was denatured for 15 min. with 50mM NaOH, 10mM NaCl and then neutralized with 0.1M Tris HC1 pH 7.4. Finally, transfer of the bands was performed in the presence of 20X SSC for 2-3 hours. Then, the membrane was cross-linked for 45 sec. in a UV cross-linker.

Hybridization:

Prehybridization:

The membranes (Northern blots) were placed in a cylinder contains 10-20 ml of prehybridization solution tightly covered with a rubber cork to prevent any evaporation or leaking and incubated at 42°C with rotation for about 2-3 hours.

Hybridization:

^{32}P labeled DNA probe ($1\text{-}2 \times 10^6$ cpm/ml of hybridization mix) and Salmon sperm DNA (100 µg/ ml of hybridization mix) were denatured by heating for 5 minutes at 100°C. These were added to prehybridization mix covering the filters and then incubated overnight at 42^0C with constant rotation. During the hybridization, the container holding the filters was tightly closed, placed in a polythene bag and sealed to prevent any leakage.

After the hybridization was completed, the container was placed upright to drain the liquid down and then with the help of a forceps, the membrane was placed in a 2x SSC buffer, facing the RNA side downwards, to prevent drying of the filter. Washing was done with 2 x SSC containing 0.1% SDS at room temperature for 10 min. and then with the same buffer at 65°C for 30 min.

The radioactivity was controlled. If it was too high, an additional washing was performed with 0.2 x SSC containing 0.1% SDS at 65°C. If the background was still high, further washing was done at high stringency conditions (0.2% SSC and *0.1%* SDS) as above.

The membrane was placed on a shining surface of the bench coat paper, covered with saran wrap and exposed to Kodak film (XOMAT AR) overnight at -70°C with an intensifying screen. Alternatively, the membrane was also exposed to phosphorimaging screen and scanned after an overnight exposure.

Long term storage of bacterial stocks:

Bacteria can be stored for many years in media containing 15% glycerol at low temperatures without significant loss of viability. A single bacterial colony was inoculated into 5-10 ml of LB medium taken in a culture flask and the culture was grown overnight. 0.85 ml of the overnight culture was transferred to a sterile vial containing 0.15 ml of sterile glycerol. The contents were mixed thoroughly by vortexing. The glycerinated cultures were then stored at -20°C in small aliquots. They can be preserved for a few years without loss of viability. Alternatively, the glycerinated suspension was stored at -70°C. Viable bacteria were recovered by

simply scratching the surface of the frozen stock with a sterile platinum loop or wire and used for streaking on agar plates or direct inoculation into liquid culture medium.

Bioinformatic tools used in this study

Multiple Sequence Alignment: Multiple sequence alignment was performed according to Combet *et al.*, (2000).

Prediction of potential glycosylation sites:

http://www.cbs.dtu.dk/services/NetNGlyc/

Restriction maps

BioEdit software

Signal peptide prediction

http://www.cbs.dtu.dk/services/SignalP-2.0/

RESULTS:

Isolation of total RNA from goat liver tissue:

Total RNA was isolated from 40 mg of goat liver tissue using RNeasy spin column (QIAgen). An aliquot of the isolated RNA was electrophoresed on 1% denaturing agarose gel. Two distinct bands corresponding to 28S rRNA and 18S rRNA were seen (Figure 23A). 28S rRNA shows double the intensity when compared to 18S rRNA. The Rf values of rRNAs and their molecular mass (in Kb and in Daltons) from different animals are shown in Table 12.

Species	rRNA	Size	MW, Daltons
Human	28S	5.0 Kb	1.7×10^6
	18S	1.9 Kb	6.1×10^5
Mouse	28S	4.7 Kb	1.57×10^6
	18S	1.9 Kb	6.1×10^5
Fish	28S	4.7 Kb	1.57×10^6
	18S	1.9 Kb	6.1×10^5
Goat	28S	4.8 Kb	1.6×10^6
	18S	1.9 Kb	6.1×10^5

Table 12: Calculated molecular mass of the 18S and 28S rRNA from goat, in comparison to the human, mouse and fish rRNA's.

GOAT MPR300 CLONING:

Primers for RT-PCR:

Degenerate primers were designed in the ligand binding domain 3 from the highly conserved regions of known MPR 300 sequence from different animal species (human, bovine mouse, rat and chicken) by multiple sequence alignment.

Primer A: 5` -GGC ATA CTC AGT GAT CCA CTC - 3' (Reverse primer)

Primer B: 5' -CTG TGC AGT TAC ACA TGG GAA GC - 3' (Forward primer)

Primer C: 5` -GCC AT AC CAC AGC TTC CCA ATT YTG - 3' (Reverse primer)

Amplification of cDNA fragments for goat MPR 300 was done by RT reaction and followed by PCR. Primers were designed to amplify cDNA fragments in the ligand binding regions i.e., 3rd domain.

About 2ug of total RNA was used for first strand synthesis employing the two MPR 300 specific reverse primers (primer A and C) in two separate reactions. One tenth of the RT product was used for the PCR amplification that was performed with HotStar Taq DNA polymerase kit using MPR 300 specific A, B and C primer. When primers A and B were used for the PCR reaction (95°C 15'; 94^{0}C 1'; 55°C 1'; 72°C 1'; 30 cycles), a fragment size corresponding to about 800bp was amplified. The gel-purified fragment was used for TA cloning into pCR 2.1 vector (Figure 23B). Positive clones were identified by blue white selection method. More than 80% of the colonies appeared white in colour (could be positive). A single white colony was picked up and inoculated into LB medium for plasmid DNA isolation and subjected to restriction digestion with EcoRI that resulted in the release of the 800 bp fragment (Figure 24A). This insert obtained from the positive clone was sequenced using vector derived M13

forward and M13 reverse primers. The nucleotide sequence obtained revealed that it displayed high degree of homology with that of other known animal species covering the partial 1st, entire 2nd and partial 3rd domains.

When primers A and C were used for PCR reaction (95°C 15'; 94°C 1'; 55°C 1'; 72°C 1'.30"; 30 cycles) using HotStar Taq DNA polymerase (QIAgen), a fragment size corresponding to about 1.3kb was amplified. The gel-purified fragment was used for TA cloning into pCR 2.1 vector. Positive clones were identified by blue white selection method. A single white colony was picked up and inoculated into LB medium for plasmid DNA isolation and subjected to restriction analysis with EcoRI that resulted in the sequence of the 1.3 kb fragment (Figure 24B). This insert was sequenced from the positive clone using vector derived M13 forward and M13 reverse primers (Figure 25). The nucleotide sequence obtained when converted into protein sequence and the result compared with the other MPR 300 sequences (mammalian and chicken) revealed that it, displayed 75-96% similarity at the amino acid level and covers the partial 1st, complete 2nd,3rd and partial 4th domains. The nucleotide sequence and the deduced amino acid sequence of this 1.3Kb fragment are shown in Figure 26. The amino acid sequence of the 1.3Kb fragment was subjected to multiple sequence alignment with other known MPR 300 proteins (Figure 27). Form the data obtained, it is evident that the goat receptor exhibited high homology in the region of the potential glycosylation sites and also the sequences connecting the repetitive domains are highly conserved through out the animal species. The arginine residue involved in ligand binding, known for other receptors is also conserved in the 3rd domain.

Northern blot analysis for goat MPR 300:

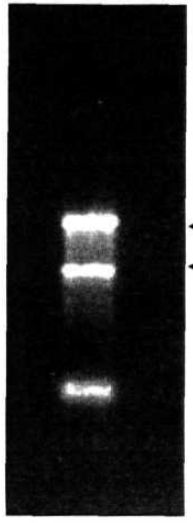
After having confirmed the sequence of the 1.3 kb fragment as goat MPR 300 cDNA, it was used as a probe for Northern blotting. 15 μg of total RNA isolated from goat liver tissue was subjected to denaturing agarose (1%) gel electrophoresis (Matzner et al., 1996) and transferred to hybond-N membrane. The membrane was exposed to UV light for 15 sec. and incubated with prehybridization buffer for a minimum of 2 hr. and then hybridized with ^{32}P-1.3 kb fragment. The membrane was washed with high stringency buffer as described under methods. A single band of about 9.3 kb was observed (Figure 28). This size of the transcript was comparable with that of the other known animal species (Table 13). Schematic diagram of partial cDNA clones obtained for goat MPR 300 in this study are shown in Figure 29.

Species	mRNA Transcript size for MPR 300	Coding sequence size
Human	~9.4 kb	7.473 kb
Bovine	~9.5 kb	7.497 kb
Mouse	~10 kb	7.446 kb
Chicken	~11.0 kb	7.410 kb
Fish	~13.5 kb	??
Goat	~9.3 kb	??

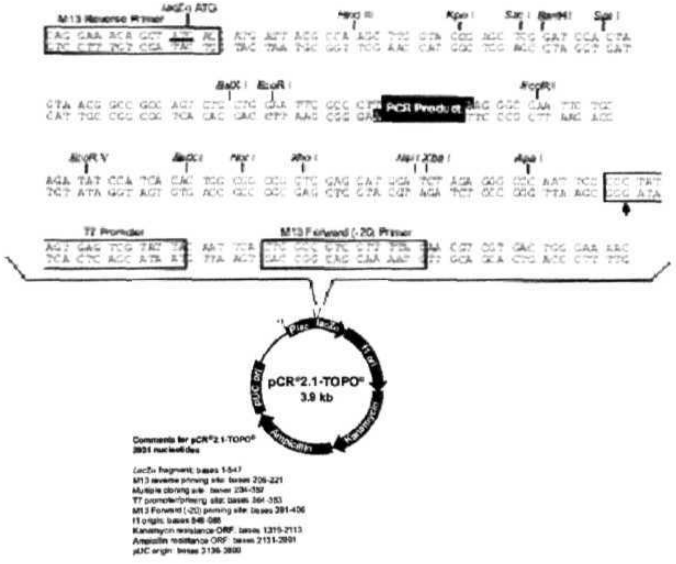
Table 13: Table showing the mRNA transcript lengths coding for MPR 300 from various species as indicated.

Figure 23: *(A) Purity and Integrity of RNA Preparation. Total RNA was isolated from goat liver tissue using the RNeasy kit. 5ug of RNA preparation was subjected to 1% denaturing agarose gel electrophoresis. Arrow indicates the position of 28S and 18S RNA.*

(B) pCR 2.1 Plasmid Vector for TA cloning (Invitrogen) The sequence represents the pCR 2.1 vector with a PCR product inserted by TA cloning which is flanked by EcoRI site on each side. Arrow indicates the start of transcription for the T7 RNA polymerase.


28S
18S

A


M13 Reverse Primer
PCR Product
T7 Promoter
M13 Forward (-20) Primer
pCR®2.1-TOPO®
3.9 kb

B

Figure 23

Figure 24: *(A) Goat total RNA was used for the first standard synthesis using* ***MPR*** *specific primer (Primer A). For the* ***PCR*** *amplification 2ul of first strand synthesis product was used along with MPR 300 specific primers (Primer A and B). Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, standard DNA ladder, Lane 2, amplified product (800bp), Lane 3, fragment released from the vector after digestion with* ***E.CoRI****.*

(B) Goat total RNA was used for the first standard synthesis using MPR specific primer (Primer C). For the amplification 2ul of first strand synthesis product was used along with MPR 300 specific primers (Primer A and C). Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, standard DNA ladder, ***Lane*** *2, amplified product* ***(1.3Kb), Lane*** *3, fragment released from the vector after digestion with E.CoRI.*

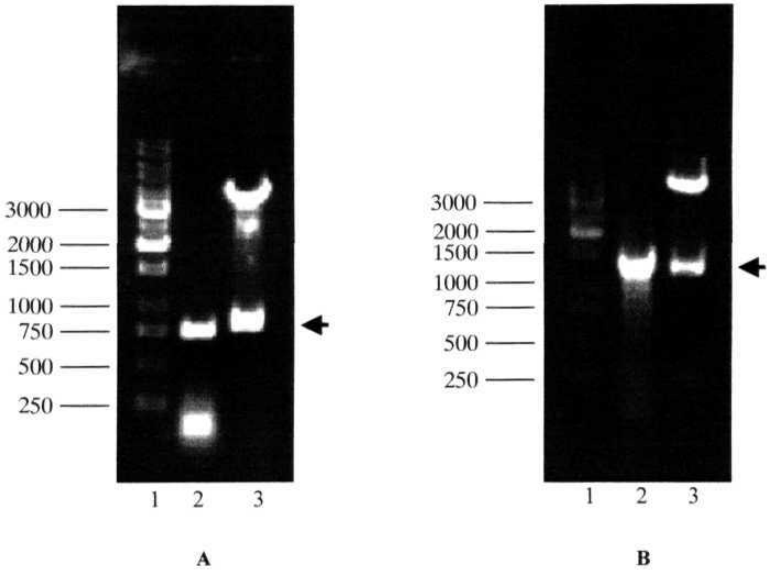
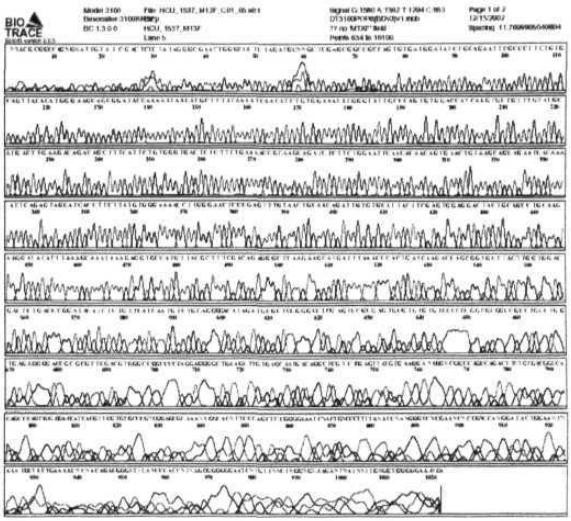
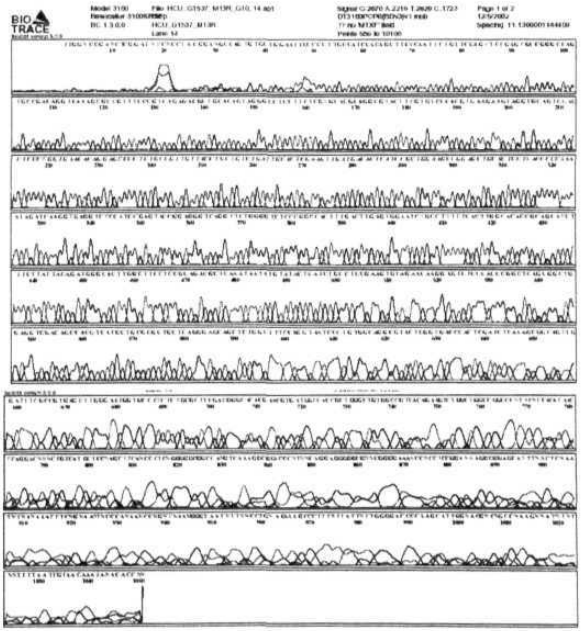

3000
2000
1500
1000
750
500
250
1 2 3
A
3000
2000
1500
1000
750
500
250
1 2 3
B

Figure 24

Figure 25: *DNA sequencing was performed on an automated DNA sequencer. (A) sequence obtained for goat MPR 300 partial cDNA clone (1.3 kb) with vector derived M13F primer. (B) sequence obtained for goat MPR 300 partial cDNA clone (1.3 kb) with vector derived M13R primer.*



A



B

Figure 25

Figure 26: *Nucleotide and deduced amino acid sequence of partial goat MPR 300 cDNA (1.3 kb).*

```
1     CTG TGC AGT TAC ACA TGG GAA GCA GCG GAT ACC AAA AAT AAC ATG   45
1      L   C   S   Y   T   W   E   A   A   D   T   K   N   N   M   15
46    CTT TAT AAA ATC AAC ATC TGT GGA AAT ATG GGT ATT GCC CAG TGT   90
16     L   Y   K   I   N   I   C   G   N   M   G   I   A   Q   C   30
91    GGA CCA TCA AGT GCT GTT TGT ATG CAT GAC TTG AAG ACA GAC AGC  135
31     G   P   S   S   A   V   C   M   H   D   L   K   T   D   S   45
136   TTT CAT TCT GTG GGT GAC TCT CTT TTG AAA ACC GCA AGC AGA TCT  180
46     F   H   S   V   G   D   S   L   L   K   T   A   S   R   S   60
181   CTT CTG GAA TTC AAC ACA ACA GTG AAC TGT AAG CAG CAG AAT CAC  225
61     L   L   E   F   N   T   T   V   N   C   K   Q   Q   N   H   75
226   AAA ATT CAG AGT AGC ATC ACC TTC TTA TGT GGG AAA ACC TTG GGA  270
76     K   I   Q   S   S   I   T   F   L   C   G   K   T   L   G   90
271   ACT CCC GAG TTT GTA ACT GCA ACA GAT TGT GTG CAT TAC TTC GAG  315
91     T   P   E   F   V   T   A   T   D   C   V   H   Y   F   E  105
316   TGG AGG ACT ACT GCA GCC TGC AAG AAG GAT ATA TTT AAA GCA AAT  360
106    W   R   T   T   A   A   C   K   K   D   I   F   K   A   N  120
361   AAA GAG GTG CCA TGT TAC GCT TTC GAC AGA GGG CTC AAG AAG CAT  405
121    K   E   V   P   C   Y   A   F   D   R   G   L   K   K   H  135
406   GAT TTA AAC CCA CTG ATC AAG ACC AGC GGT GCT TAC TTG GTG GAC  450
136    D   L   N   P   L   I   K   T   S   G   A   Y   L   V   D  150
451   GAC TCT GAC CCG GAT ACA TCT CTG TTC ATC AAT GTC TGC AGG GAC  495
151    D   S   D   P   D   T   S   L   F   I   N   V   C   R   D  165
496   ATA GAT GCG CTC CGG GCC TCG AGT CCG CGA GTG CGT GTG TGT CCC  540
166    I   D   A   L   R   A   S   S   P   R   V   R   V   C   P  180
541   CCC GGC GCG GCC GCC TGC CTG GTG AGA GGG GAC CGC GCG TTC GAC  585
181    P   G   A   A   A   C   L   V   R   G   D   R   A   F   D  195
586   GTG GGC CGG CCC CAG GAG GGG CTG AAG CTT GTG AGC AAT GAC AGG  630
196    V   G   R   P   Q   E   G   L   K   L   V   S   N   D   R  210
631   CTC GTC TTG AGT TAT GTG AAG GAG GGG GCC GGC CAG CCA GAC TTC  675
211    L   V   L   S   Y   V   K   E   G   A   G   Q   P   D   F  225
676   TGT GAC GGC CAC AGC CCA GCG GTG ACC ATC ACG TTC GTG TGC CCG  720
226    C   D   G   H   S   P   A   V   T   I   T   F   V   C   P  240
721   TCG GAG CGC AGA GAG GGC ACC ATT CCC AAG CTC ACG GCG AAA TCC  765
241    S   E   R   R   E   G   T   I   P   K   L   T   A   K   S  255
766   AAC TGC CGC TTT GAG ATC GAG TGG GTC ACC GAG TAC GCC TGC CAC  810
256    N   C   R   F   E   I   E   W   V   T   E   Y   A   C   H  270
811   AGG GAT TAC CTG GAA AGC CAG AGC TGC TCC CTG AGC AGC GCG CAG  855
271    R   D   Y   L   E   S   Q   S   C   S   L   S   S   A   Q  285
856   CAT GAC GTG GCT GTC GAC CTC CAG CCG CTG AGC CGG GTG GGA GAC  900
286    H   D   V   A   V   D   L   Q   P   L   S   R   V   G   D  300
901   TCC TTG TTC TAC ACT TCG GAG GCA GAT GAG TAT ACA TAT TAT TTG  945
301    S   L   F   Y   T   S   E   A   D   E   Y   T   Y   Y   L  315
946   AGC GTC TGC GGA GGA AGC CAA GTG CCC ATC TGT AAT AAG AAA GAT  990
316    S   V   C   G   G   S   Q   V   P   I   C   N   K   K   D  330
991   GCT GCG GTG TGC CAA GTG AAA AAG GCA GAT TCC ACT CAA GTC AAA 1035
331    A   A   V   C   Q   V   K   K   A   D   S   T   Q   V   K  345
1036  GTG GCC GGG AGA CCC CAG AAC CTG ACC CTC CGG TAC TCG GAT GGA 1080
346    V   A   G   R   P   Q   N   L   T   L   R   Y   S   D   G  360
1081  GAC CTC ACC TTG ATC TAT TTT GGG GGT GAG GAG TGC AGC TCC GGC 1125
361    D   L   T   L   I   Y   F   G   G   E   E   C   S   S   G  375
1126  TTC CAG CGG ATG AGT GTC ATC AAC TTC GAG TGC AAT CAG ACA GCA 1170
376    F   Q   R   M   S   V   I   N   F   E   C   N   Q   T   A  390
1171  GGT AAC AAC GGC AGA GGG GCT CCT GTG TTC ACC GGG GAG GTG GAC 1215
391    G   N   N   G   R   G   A   P   V   F   T   G   E   V   D  405
1216  TGC ACC TAC TTC TTC ACG TGG GAC ACG AAG TAC GCC TGC GTG CAC 1260
406    C   T   Y   F   F   T   W   D   T   K   Y   A   C   V   H  420
1261  GAG AAG GAG GCC CTG CTG TGC AGC GTC TCT GAC GGG AAA CAG CGC 1305
421    E   K   E   A   L   L   C   S   V   S   D   G   K   Q   R  435
1306  TTT GAC CTG TCG GCA CTG GCC CGG CAC TCA GAA CTG GAA CAG AAT 1350
436    F   D   L   S   A   L   A   R   H   S   E   L   E   Q   N  450
1351  TGG GAA GCT GTG GAT GGC   1368
451    W   E   A   V   D   G
```

Figure 26

Figure 27: *Alignment of the amino acid sequence of 1.3Kb fragment of goat MPR 300. From the partial cDNA sequence of goat MPR 300, domains 1-4 were having conserved motif DLSXL at the carboxyterminal border of each domain. Domains were aligned by the clustalw method.*

** indicates the conserved residues.*

The region between domain and domain is represented in yellow shade.
The potential glycosylation sites are represented in brown shade.
The arginine residue involved for ligand biding is represented in blue shade.

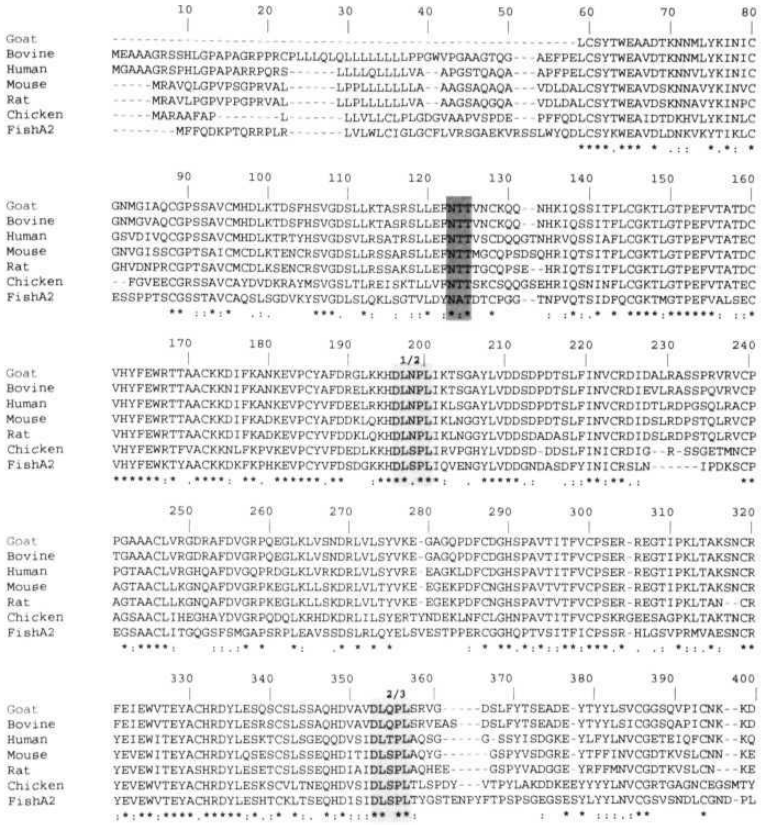


Figure 27

Figure 28: *Northern Blot Analysis. Goat MPR 300 mRNA transcript detected by Northern blot analysis. 15ug of total RNA isolated for goat liver tissue and subjected to denaturing 1% agarose gel electrophoresis, transferred to hybond-N nylon membrane and hybridized with* ^{32}P *labeled goat MPR 300 specific cDNA fragment (1.3Kb). A single band corresponding to ~9.3Kb mRNA transcript was obtained.*

Figure 29: *(A) Schematic diagram of bovine MPR 300 gene (B) Schematic diagram represents the partial clone (800bp) obtained for goal MPR 300 (C) Schematic diagram represents the partial clone (1.3 kb) obtained for goat MPR 300.*

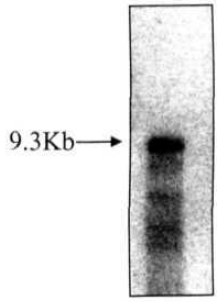
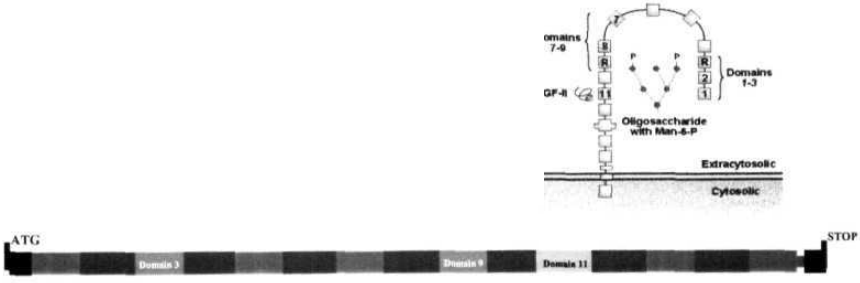
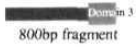


Figure 28

(A)



(B)



800bp fragment

(C)

1.3Kb fragment

Figure 29

GOAT MPR46 CLONING:

Primers for RT-PCR:

Degenerate primers were designed from the highly conserved regions of known sequences of MPR 46 from different animal species (human, bovine mouse, rat and chicken) by multiple sequence alignment.

Primer 1:5'- TCA CAT TGG TAA YAA RTG RTG ATC - 3' (Reverse primer).

Primer 2: 5' - GTG GCA GTS AGA GAA TCY TGG CAG - 3' (Forward primer).

Primer 3: 5' - ATG ATG TCC CCC CTC CAC AGC TCC - 3' (Forward primer).

Amplification of cDNA fragments for goat MPR 46 was done by RT, followed by PCR About 1 -2 μg of total RNA was used for first strand synthesis performed with MPR 46 specific reverse primer 1. One tenth of the RT product was used for the PCR amplification which was performed with HotStar Taq DNA polymerase kit using MPR 46 specific primers (primer 1, 2 and 3). When primers 1 (reverse) and 2 (forward) were used for the PCR reaction (95°C 15'; 94°C 1'; 55°C 1'; 72°C 1'; 30 cycles), a fragment size corresponding to about 760bp was amplified. The gel purified fragment was used for TA cloning into pCR 2.1 vector as done for MPR 300. Plasmid DNA was isolated from the positive colonies and subjected to restriction digestion with EcoRI that resulted in the release of 760 bp fragment (Figure 30A). This insert was sequenced from the positive clone using vector derived M13 forward and M13 reverse primers. The nucleotide sequence obtained revealed that the 760bp fragment obtained displayed 90-98% similarity at the amino acid level with the corresponding mammalian and partial chicken MPR 46. This cDNA clone covers partial goat MPR 46 sequence.

When primers 1 and 3 were used for the PCR reaction (95°C 15'; 94°C 1'; 55°C 1'; 72^0C 1'; 30 cycles), a full length clone for MPR 46 (840bp) was amplified. The gel purified fragment was used for TA cloning into pTZ57R vector (MB1 Fermentas). Positive clones were identified by blue white selection. More than 80% of the colonies appeared white in colour (could be positive) and the rest blue in colour. A single white colony was picked up and inoculated into LB medium for plasmid DNA isolation and subjected to restriction analysis with *PstI* and *KpnI* (Figure 30B). The sequence of the vector is shown in (Figure 30C). The sequence of the full length clone was obtained using vector derived M13 forward and M13 reverse primers (Figure 31). The nucleotide sequence obtained for the 840 bp fragment revealed that it displayed 87-97% similarity at the amino acid level with the corresponding mammalian and chicken MPR 46 and covers the full length MPR 46 gene including start and stop codon. The nucleotide and deduced amino acid sequence of this full length cDNA clone is shown in Figure 32. Figure 33 shows the multiple sequence alignment of goat MPR 46 to other known MPR sequences. From the results it is evident that the sequences of goat receptor exhibit high degree of homology especially at putative potential glycosylation sites (five). The arginine and histidine residues which are known to be involved in ligand binding and the sequences adjacent to these, the transmembrane region and the cytoplasmic tail are highly conserved.

*Northern **blot** analysis **for** goat **MPR** 46:*

After having confirmed the sequence of the full-length cDNA clone for goat MPR 46, it was used as a probe for Northern blotting. 15 μg of total RNA isolated from goat liver tissue was subjected to denaturing agarose (1%) gel electrophoresis (Matzner et al., 1996) and then transferred on to hybond-N membrane. The membranes were exposed

to UV light for 15 sec., incubated with prehybridization buffer for a minimum of 2 hr and then hybridized with ^{32}P-full length cDNA clone for MPR 46. The membrane was washed with high stringency buffer as described under methods. A single band of about 2.3kb was observed (Figure 34). This size of the transcript was comparable with that of the other known animal species (mouse 2.3 kb and chicken 3.3 kb).

Figure 35 shows a schematic diagram for partial and full length clones for goat MPR 46 obtained in this study.

Figure 30: *(A) Goat total RNA was used for the first standard synthesis using* ***MPR*** *specific primers (Primer 2). For the* ***PCR*** *amplification of partial cDNA clone, 2ul of first strand synthesis product was used along with MPR 46 specific primers (Primer J* ***and*** *2). Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, standard DNA ladder, Lane 2, amplified product (760 bp), Lane 3, fragment released from the vector after digestion with* ***ECoRI****.*

(B) For the PCR amplification of full length clone, 2ul of first strand synthesis product was used along with MPR 46 specific-primers (Primer 2 and 3). Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, standard DNA ladder, Lane 2, amplified product (840bp), Lane 3, fragment released from the vector after digestion with ***KpnI*** *and* ***Pst*** *I.*

(C) pTZ57R ***plasmid*** *vector for TA cloning (****MBI, Fermentas****). The sequence represents the pTZ57R plasmid, PCR product insert at* ***Eco321*** *site.*

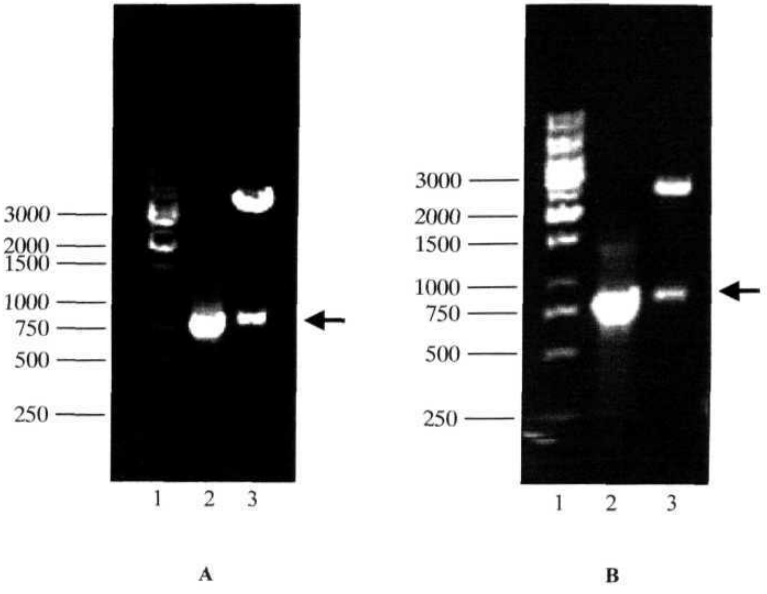
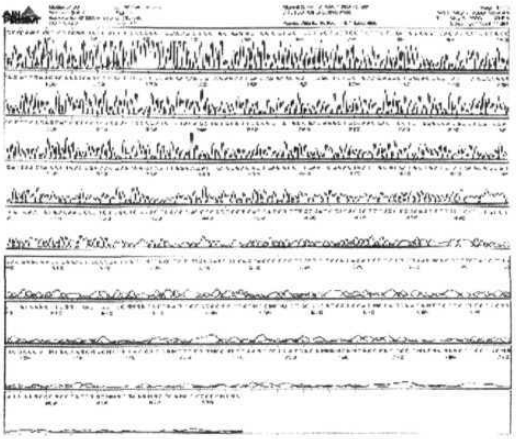
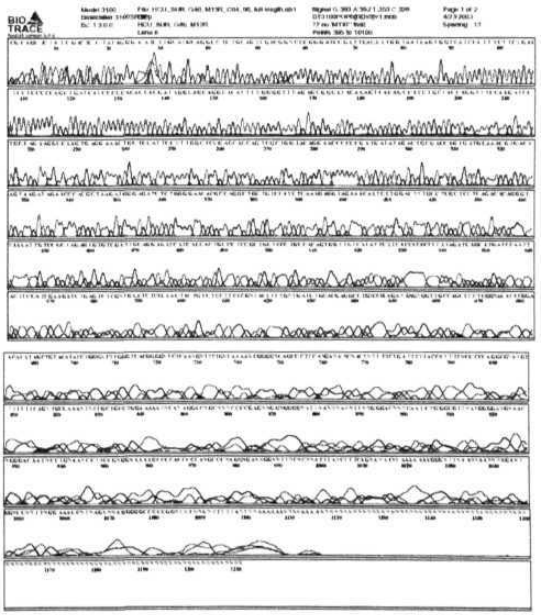

3000
2000
1500
1000
750
500
250
1 2 3
A
3000
2000
1500
1000
750
500
250
1 2 3
B
pTZ57R
2886 bp
f1 (M)
bla (Ap^r)
rep (pMB1)
MCS
lacZ
GAATTCGAGCTCGGTACCTCGCGAATGCATCTAGAT
ATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTT
615
695

Figure 30

Figure 31: *DNA sequencing was performed on an automated DNA sequencer. (A) sequence obtained for goat MPR 46 full length cDNA clone (840 bp) with vector dervied M13F primer. (B) sequence obtained for goat MPR 46 full length clone (840 bp) with vector derived M13R primer.*



A



B

Figure 31

***Figure** 32: Nucleotide and deduced amino acid sequence of goat MPR 46 full length cDNA clone.*

***Figure 33:** Alignment of the amino acid sequence of full length goat MPR 46. Amino acids were aligned by clustalw method.*

** indicates the conserved residues.*

Potential glycosylation sites are shown in the brown shade.

*Under lined area (magenta shade) represents the **transmembrane** domain.*

$ (red shade) critical histidine and arginine residues involved in ligand biding.

```
1    ATG ATG TCC CCC CTC CAC AGC TCC TGG AGG ACT GGC CTG CTC CTG   45
1     M   M   S   P   L   H   S   S   W   R   T   G   L   L   L   15
46   CTG CTG CTC TTC TCC ATG GCA GTC AGA GAA TCT TGG CAG ACT GAA   90
16    L   L   L   F   S   M   A   V   R   E   S   W   Q   T   E   30
91   GAG AAA ACA TGC GAC CTG GTG GGA GAA AAG GGT AAA GAA TCA GAG  135
31    E   K   T   C   D   L   V   G   E   K   G   K   E   S   E   45
136  AAA GAG TTG GCT CTC CTG AAG AGG CTG ACA CCG CTA TTT AAC AAA  180
46    K   E   L   A   L   L   K   R   L   T   P   L   F   N   K   60
181  AGC TTT GAG AGC ACC GTG GGC CAG AGC CCA GAT ATG TAC AGC TAT  225
61    S   F   E   S   T   V   G   Q   S   P   D   M   Y   S   Y   75
226  GTG TTC CGG GTG TGC CGA GAA GCT GGC AAC CAC TCC TCT GGG GCA  270
76    V   F   R   V   C   R   E   A   G   N   H   S   S   G   A   90
271  GGC CTC GTG CAG ATC AAC AAA AGT AAC GGG AAG GAG ACA GTA GTT  315
91    G   L   V   Q   I   N   K   S   N   G   K   E   T   V   V  105
316  GGG AGA TTC AAC GAG ACT CAG ATC TTC AAT GGA AGT AAT TGG ATC  360
106   G   R   F   N   E   T   Q   I   F   N   G   S   N   W   I  120
361  ATG CTG ATC TAT AAA GGG GGT GAT GAA TAT GAC AAC CAC TGT GGC  405
121   M   L   I   Y   K   G   G   D   E   Y   D   N   H   C   G  135
406  AGG GAG CAG CGG AGG GCA GTG GTG ATG ATC TCC TGC AAT CGA CAC  450
136   R   E   Q   R   R   A   V   V   M   I   S   C   N   R   H  150
451  ACT CTA GCG GAC AAT TTT AAC CCT GTG TCT GAG GAG CGA GGC AAA  495
151   T   L   A   D   N   F   N   P   V   S   E   E   R   G   K  165
496  GTC CAA GAT TGT TTC TAC CTC TTT GAG ATG GAC AGC AGC CTG GCG  540
166   V   Q   D   C   F   Y   L   F   E   M   D   S   S   L   A  180
541  TGT TCC CCA GAG ATC TCC CAT CTT AGC GTG GGT TCT ATC TTA CTT  585
181   C   S   P   E   I   S   H   L   S   V   G   S   I   L   L  195
586  GTC ACG TTT GCA TCA CTG GTC GCA GTC TAT ATC ATC GGG GGG TTC  630
196   V   T   F   A   S   L   V   A   V   Y   I   I   G   G   F  210
631  CTG TAC CAG CGA CTG GTG GTC GGA GCC AAA GGA ATG GAG CAG TTT  675
211   L   Y   Q   R   L   V   V   G   A   K   G   M   E   Q   F  225
676  CCT CAC TTG GCC TTC TGG CAG GAT CTT GGA AAC CTG GTA GCA GAT  720
226   P   H   L   A   F   W   Q   D   L   G   N   L   V   A   D  240
721  GGC TGT GAC TTT GTA TGC CGC TCT AAA CCC CGA AAT GTG CCT GCT  765
241   G   C   D   F   V   C   R   S   K   P   R   N   V   P   A  255
766  GCC TAT CGT GGT GTG GGG GAT GAT CAG CTG GGG GAG GAG TCA GAA  810
256   A   Y   R   G   V   G   D   D   Q   L   G   E   E   S   E  270
811  GAA AGG GAT GAC CAC TTA TTA CCA ATG TGA   840
271   E   R   D   D   H   L   L   P   M   *
```

Figure 32

```
                10        20        30        40        50        60
                |         |         |         |         |         |
Goat            MMSPLHSSWRTGLLLLLLFSMAVRESWQTEEKTCDLVGEKGKESEKELALLKRLTPLFNK
Bovine          MMSPLHSSWRTGLLLLLLFSVAVRESWQTEEKTCDLVGEKGKESEKELALLKRLTPLFNK
Human           -MFPFYSCWRTG-LLLLLLAVAVRESWQTEEKTCDLVGEKGKESEKELALVKRLKPLFNK
Mouse           -MFPFSGCWRTELLLLLLLAVAVRESWQIEEKSCDLVGEKDKESKNEVALLERLRPLFNK
Chicken         ------------------------------------------------------------

                70        80        90        100       110       120
                |         |         |         |         |         |
Goat            SFESTVGQSPDMYSYVFRVCREAGNHSSGAGLVQINKSNGKETVVGRFNETQIFNGSNWI
Bovine          SFESTVGQSPDMYSYVFRVCREAGNHSSGAGLVQINKSNGKETVVGRFNETQIFNGSNWI
Human           SFESTVGQGSDTYIYIFRVCREAGNHTSGAGLVQINKSNGKETVVGRLNETHIFNGSNWI
Mouse           SFESTVGQGSDTYSYIFRVCREASNHSSGAGLVQINKSNDKETVVGRINETHIFNGSNWI
Chicken         ------------------------------------------------------------

                130       140       150       160       170       180
                |  $      $|        |         |         |         |
Goat            MLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFYLFEMDSSLA
Bovine          MLIYKGGDEYDNHCGREQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFYLFEMDSSLA
Human           MLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLADNFNPVSEERGKVQDCFYLFEMDSSLA
Mouse           MLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLAANFNPVSEERGKVQDCFYLFEMDSSLA
Chicken         ----------------------IPDVSS--------FSIISEEREKEQDCFYLFEMDSSVA
                                       :  :*.         *. :**** * ***********:*

                190       200       210       220       230       240
                |         |         |         |         |         |
Goat            CSPEISHLSVGSILLVTFASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVAD
Bovine          CSPEISHLSVGSILLVTLASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVAD
Human           CSPEISHLSVGSILLVTFASLVAVYVVGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVAD
Mouse           CSPEVSHLSVGSILLVIFASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVAD
Chicken         CPAEDSHLSTGSILLITFSALVTVYIVGGFLYQRLIVGAKGMEQFPHFAFWQDLGNLVAD
                *..* ****.*****: :::**:**::********:**********:***********

                250       260       270
                |         |         |
Goat            GCDFVCRSKPRNVPAAYRGVGDDQLGEESEERDDHLLPM
Bovine          GCDFVCRSKPRNVPAAYRGVGDDQLGEESEERDDHLLPM
Human           GCDFVCRSKPRNVPAAYRGVGDDQLGEESEERDDHLLPM
Mouse           GCDFVCRSKPRNVPAAYRGVGDDQLGDESEERDDHLLPM
Chicken         GCDFVCRSKPRNVPAAYRGVGDDQLGDESEERDDHLLPM
                ************************:************
```

Figure 33

Figure 34: *Northern blot analysis: Goat MPR 46 **mRNA** transcript detected by Northern blot analysis. 15ug of total RNA isolated from goat liver tissue and subjected to 1% denaturing agarose gel electrophoresis, transferred to **hybond-N** nylon membrane and hybridized with ^{32}P labeled goat MPR 46 specific cDNA fragment (8()<)bp). A single hand corresponding to ~2.3Kh **mRNA** transcript was obtained.*

***Figure** 35: (A) Schematic diagram of bovine MPR 46 gene.*

***(B)** Schematic diagram of partial goat MPR 46 cDNA clone obtained with primers 1 and 2.*

(C) Schematic diagram of full length goat MPR 46 cDNA clone obtained with primers 1 and 3.

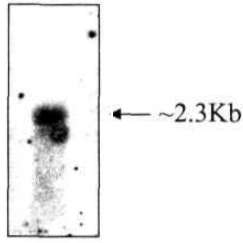


Figure 34

Figure 35

DISCUSSION:

The present study describes for the first time the isolation and characterization of a partial cDNA clone for goat MPR 300 and a full length cDNA clone for goat MPR 46 protein.

To obtain these informations, RT-PCR experiments were performed. In the first instance, several primers were designed based on multiple sequence alignment of known MPR protein sequences.

Molecular cloning of goat MPR 300:

The known MPR 300 cDNA sequences are in the range of 7-7.4kb (Table 14). In the present study, an attempt was made to obtain a partial cDNA clone for the goat MPR 300 spanning the initial domain structures in the extracytoplasmic region of the receptor because of the recent finding that the fish receptor exhibits extensive sequence homology to the mammalian and chicken receptors in the extracytoplasmic region (Udaya Lakshmi *et al.,* 2000). Initially, using the total RNA from the liver tissue, first strand cDNA synthesis was accomplished using the primers designed (A and C). This RT product was then amplified by PCR and the product (800bp) cloned into the vector. From the sequence results obtained for this, it is clear that this fragment represents the MPR 300 cDNA.

Additional sequence information was obtained by using the primer designed (primer A and Primer B) in a PCR reaction. From the sequence results obtained, it is clear that the cDNA isolated is the putative cDNA for MPR 300.

Several lines of evidences support that the partial cDNA clone obtained in this study indeed represents the goat MPR 300 protein.

1. The amino terminal portion of the sequence obtained is divided into domains which show similar length as their chicken and mammalian counterparts.

2. The carboxyl terminal border of each cassette is defined by the conserved motif DLS(P/R/S)L which strongly resembles the corresponding sequences found in mammalian and chicken MPRs (DLXXL).

3. All the conserved cysteines which have been suggested to form disulfide bonds in bovine MPR 300 (Lobel *et al,* 1988) and present in all vertebrate species studied so far were also seen in the goat receptor.

4. Three glycosylation sites were found in partial 1st and complete 2nd and 3rd and partial 4th domain sequences.

5. Domains 3 and 9 of bovine MPR 300 gene have been shown to contain two independent binding sites for mannose 6-phosphate carrying ligands. Binding of mannose 6-phosphate in both cases depends on a critical arginine residue (R435 in domain 3 and R1334 in domain 9, Dahms *et al,* 1993). Further, recent partial cDNA analysis of the fish MPR 300 also reveals the presence of the critical arginine residue in domain 3 (Udaya Lakshmi et al., 2000) that is important for ligand binding. The sequence obtained for goat MPR 300 reveals the presence of a corresponding arginine residue (R 435) in domain 3, which is surrounded by the highly conserved sequence motif CSSGFQRM (S/T)(V/I)INF (E/Q)C.

Table 14 summarizes the similarity in various regions of the goat MPR 300 to other known MPR 300 proteins.

Species	Size of mature protein (kDa)	cDNA clone size (kb)	Repetitive domain sequence	Conservation of cystein residues	Ligand binding region sequences (in 3rd domain)	Potential glycosylation sites	Size of RNA transcript (kb)
Bovine	300	7.497	DL(N/Q)(P/A)L	Yes	CSSCHQRMSV	Conserved	~9.5
Human	300	7.473	DL(N/T/S)(P/A)L	Yes	CSSGFQRMSV	Conserved	~9.4
Mouse	300	7.446	DL(N/S)(P/V)L	Yes	CSSGFQRMSV	Conserved	~10.0
Rat	300		DL(N/S)(P/V)L	Yes	CSSGFQRMSV	Conserved	
Goat	300	??	DL(N/Q/S)(P/A)L	Yes	CSSGFQRMSV	Conserved	~9.3
Chicken	300	7.410	DLSPL	Yes	CSSGFQRMTV	Conserved	~11.0
Fish	300	??	DLS(P/R)L	Yes	CSSGFQRMTI	Conserved	~13.5

Table 14: Similarity in various regions of the goat MPR 300 to other known MPR 300 proteins.

Taken together, these data suggest that the partial cDNA sequence presented here encodes for goat MPR 300.

Molecular cloning of goat MPR 46:

The known MPR 46 cDNA sequences are in the range of 830-840bp(Dahms and Hancock, 2002). In the present study, first an attempt was made to obtain a partial cDNA clone for the goat MPR 46 spanning the extracytoplasmic region of the receptor. Initially, using the total RNA from the liver tissue, first strand cDNA synthesis was accomplished using the primer 1. This RT product was then amplified by PCR (primers 1 and 2) and the product (760bp) cloned into the vector. From the sequence results obtained for the partial cDNA clone, it is clear that the fragment (760bp) represents the MPR 46 cDNA.

Additional sequence information was obtained for a full length clone by using the primer 1 and primer 3 in the PCR reaction. From the sequence results obtained, it is clear that the cDNA isolated is the putative cDNA for MPR 46.

The most important observations and conclusions that can be drawn from the results are

i) there is a striking homology of the conserved cytoplasmic tail sequences including the transport signals that have been identified in human MPR 46 in the goat protein.

ii) the cysteine residues that aid in disulfide pairing in the amino terminal domain of the receptor are highly conserved.

iii) the transmembrane domain is highly conserved.

iv) five potential glycosylation sites are seen.

v) the predicted arginine residue in the ligand binding region of other known MPRs is also seen in goat MPR 46.

Thus, the present study provides the first evidence to show that the goat MPR 46 sequences are homologous to other known mammalian MPR 46 sequences and to the partial chicken MPR 46 sequences (Matzner *et al.*, 1996), suggesting that this receptor in mammals is highly conserved. However, the function of the goat MPR 46 receptor remains to be established. This can be achieved by expression of goat cDNA into MPR minus cells and by looking at the sorting of lysosomal enzymes.

From literature it is also known that the mammalian MPR 300 and 46 sequences exhibit homology with each other in the extracytoplasmic region (Udaya Lakshmi 2000). The extracytoplasmic domain of MPR 300 is comprised of 15 repetitive units which share

significant sequence similarity with each other and also with the single unit that constitutes the extracytoplasmic domain of MPR 46. Further the repetitive domains of known MPR 300 proteins also exhibit 14-38% sequence homology (Hille-Rehfeld, 1995). The extracytoplasmic domain sequence of goat MPR 46 also exhibits 26-28% homology with bovine, human and mouse CI-MPR proteins. The available sequences of MPR proteins among a wide variety of animal species including humans indicates that these proteins are highly conserved in evolution, apparently to perform specific *in vivo* functions though these have not been defined. Only a partial sequence of the fish MPR 300 protein is published (Udaya Lakshmi *et al*., 2000). Recent biochemical and immunological studies revealed the presence of both putative receptors in the invertebrate mollusc (Siva Kumar and von Figura, 2002). It would be interesting to obtain complete sequence informations for the fish receptors and that of mollusc receptors, to establish the evolutionary pattern of both the receptors in the animal kingdom. Subsequent analysis of their functions in non-mammalian vertebrates and invertebrates should provide useful information on structure-function relationships of these interesting proteins. Current work in our laboratory is focussed on these lines.

SUMMARY

SUMMARY

- The affinity matrix Sepharose-divinyl sulfone-Phosphomannan gel was used to purify the goat **MPR** 300 and MPR 46. Further, this gel was used to purify both the MPR proteins from chicken liver tissue.
- Polyclonal antibodies were raised for goat MPR 300 and MPR 46 proteins. These antibodies have been shown to cross-react with chicken **MPR** 300 and MPR 46 proteins, respectively. Moreover, an MSC1 antibody (raised against synthetic peptide of human MPR 46 cytoplasmic tail) has been shown to cross-react with both goat and chicken MPR46 proteins.
- The ELISA method developed in this study is useful to quantify the MPRs at ng level from the membrane extracts of tissues. This method can be extrapolated to quantify the levels of MPRs in different tissues during developmental stages.
- The Immuno-affinity method developed in this study is useful to purify MPRs in large quantities with high purity in a single step, thus avoiding the use of the expensive mannose 6-phosphate.
- Chemical modification studies unveil that the arginine and histidine residues are important for the ligand binding property of goat and chicken **MPR** 46 proteins.
- Purified goat and chicken MPR 300 proteins can bind to human IGF-II. This was demonstrated by crosslinking, immunoprecipitation of the cross-linked product and the more sensitive ligand blot assay with biotinylated IGF-II. In addition to this, chicken embryonic fibroblast cells can bind ^{125}I-IGF-II and can internalize the same.
- The partial cDNA sequence obtained for goat **MPR** 300, reported here for the first **time** consists of partial 1st, complete 2nd, 3rd and partial 4th domain. The amino acid sequence of goat **MPR** 300 displayed 75-96% similarity with mammalian and

chicken MPR 300. Particularly, all the cysteine residues involved in the disulfide boding were conserved. The arginine residue in domain 3 which has been shown to be critical for mannose 6-phosphate binding in bovine MPR 300 was also found in goat MPR 300. The peptide motif CSSGFQRM in the neighborhood of this arginine was found to be identical in all mammalian and non-mammalian MPR 300 proteins studied so far. In Northern blot, the size of the mRNA transcript encoding goat MPR 300 was estimated as ~9.3 kb.

- A full-length cDNA clone obtained for goat MPR 46 reported here for the first time consists of the initiator ATG, an open reading frame and a stop codon TGA. The amino acid sequence of goat MPR 46 displayed 90-98% similarity with mammalian and partial chicken MPR 46 sequence. All the cysteine residues involved in disulfide bonding were found to be conserved. The arginine and histidine residues which are known to be involved in ligand binding in mammals, are also seen in the goat MPR 46 sequence. The cytoplasmic tail and transmembrane regions are highly conserved. In addition, five potential glycosylation sites are shown in the full-length goat MPR 46. In Northern blot, the size of the mRNA transcript encoding goat MPR 46 was estimated as ~2.3 kb.

BIBLIOGRAPHY

Admiraal,P.J., van Kesteren,C.A., Danser,A.H. Derkx,F.H., Sluiter,W. and Schalekamp,M.A. (1999). Uptake and proteolytic activation of prorenin by cultured human endothelial cells. J. Hypertens. 17: 621-629.

Aeed,P.A., Guido,D.M., Mathews,W.R. and Elhammer,A.P. (1992). Characterization of the oligosaccharide structures on recombinant human prorenin expressed in Chinese hamster ovary cells. Biochemistry. 31: 6951-6961.

Alwine,J.C., Kemp,D.J. and Stark,G.R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci U S A. 74: 5350-5354.

Amri,K., Freund, N., Vilar, J., Merlet-Benichou, C. and Lelievre-Pegorier, M. (1999). Adverse effects of hyperglycemia on kidney development in rats: *in vivo* and *in vitro* studies. Diabetes. 48: 2240-5.

Amri,K., Freund, N., Van Huyen, J.P., Merlet-Benichou, C. and Lelievre-Pegorier, M. (2001). Altered nephrogenesis due to maternal diabetes is associated with increased expression of IGF-II/mannose 6-phosphate receptor in the fetal kidney. Diabetes. 50: 1069-1075.

Anderson,R.G.W., (1993). Dissecting clathrin-coated pits. Trends Cell Biol. 3, 177-179.

Ansorge,W. (1985). Fast and sensitive detection of proteins and DNA bands by treatment with potassium permanganate. J.Biochem.Biophys.Meth. 11: 13-20.

Bach,G., Bargal, R. and Cantz, M. (1979). I-cell disease: deficiency of extracellular hydrolase phosphorylation. Biochem. Biophys. Res. Comm. 91: 976-81.

Barbero,P., Bittova, L. and Pfeffer, S.R. (2002). Visualization of Rab9-mediated vesicle transport from endosomes to the *trans*-Golgi in living cells. J. Cell Biol. **156**: 511-518.

Bergman,L.W. and Kuehl, W.M. (1978). Temporal relationship of translation and glycosylation of immunoglobulin heavy and light chains. Biochemistry 17: 5174-5180.

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

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.

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

Boman,A.L., Zhang,C, Zhu,X. and Kahn,R.A.. (2000). A family of ADP-ribosylation factor effectors that can alter membrane transport through the *trans*-Golgi. Mol Biol Cell. 11:1241-1255.

Bonner,W.M. and Laskey,R.A. (1974). A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur.J.Biochem. **46:** 83-88.

Bretthauer,R.K., Kaczorowski,G.J. and Weise, MJ. (1973). Characterization of phosphorylated pentasaccharide isolated from *Hansenula holstii* NRRL Y-2448 phosphonomannan. Biochemistry. 12: 1251-1256.

Brown,W.J. and Farquhar,M.G (1984a). The mannose 6-phosphate receptor for lysosomal enzymes is concentrated in cis-Golgi cisternae. Cell. 36: 295-307.

Brown,W.J. and Farquhar,M.G (1984b). Accumulation of coated vesicles bearing mannose 6-phosphate receptors for lysosomal enzymes in the Golgi region of I-cell fibroblasts. Proc.Nat.Acad.Sci.USA. 81:5135-5139.

Brunetti,C.R., Burke,R.L., Kornfeld,S., Gregory,W., Masiarz,F.R., Dingwell,K.S. and Johnson,D.C. (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,C.R., Dingwell,K.S., Wale,C., Graham,F.L. and Johnson,D.C. (1998). Herpes simplex virus gD and virions accumulate in endosomes by mannose 6-phosphate-dependent and independent mechanisms. J.Virol. 72: 3330-3339.

Burkhardt,J.K., 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,J.C., Devi, G.R., De Souza, A.T., Jirtle, J.L. and MacDonald, R.G.. (1999). Disruption of ligand binding to the insulin-like growth factor II/mannose 6-phosphate receptor by cancer-associated missense mutations. J. Biol. Chem. **274:** 24408-24416.

Cacia,J.,Cynthia,P.Quan., Roger Pai. and John Frenz. (1998). Human DNase 1 contains mannose 6-phosphate and binds the cation-independent mannose 6-phosphate receptor. Biochemistry. 37: 15154-15161.

Canfield,W.M. and Kornfeld,S. (1989). The chicken liver cation-independent mannose 6-phosphate receptor lacks the high affinity binding site for insulin-like growth factor II. J.Biol.Chem. **264:** 7100-7103.

Canfield,W.M, Johnson,K.F., Ye,R.D., 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.

Carroll,K.S., Hanna, J., Simon, I., Krise, J., Barbero, P. and Pfeffer, S.R. (2001). Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. Science. **292:** 1373-1376.

Causin,C., Waheed,A., Braulke,T., Junghans,U., Maly,P., Humbel,R.E. and von Figura,K. (1988). Mannose 6-phosphate/insulin-like growth factor II binding proteins in human serum and urine: their relation to the mannose 6-phosphate /insulin-like growth factor II receptor. Biochem.J. **252:** 795-799.

Chen,H.J., Remmler,J., Delaney,J.C., Messner,D.J. and Lobel,P. (1993). Mutational analysis of the cation-independent mannose 6-phospahte/insulin-like growth factor II receptor. A consensus casein kinase II site followed by 2 leucines near the carboxy terminus is important for intracellular targeting of lysosomal enzymes. J.Biol.Chem. **268;** 22338-46.

Clairmont,K.B. and Czech,M. (1989). Chicken and *xenopus* mannose 6-phosphate receptors fails to bind insulin-like growth factor II . J.Biol.Chem. **264:** 16390-16392.

Combet,C, Blanchet,C, Geourjon,C. and Deleage,G. (2000). Trends Bioche.Sci. **25:** 147-150.

Corvera,S. and Czech,M.P. (1985). Mechanism of insulin action on membrane protein recycling: a selective decrease in the phosphorylation state of insulin-like growth factor II receptors in the cell surface membrane. Proc Natl Acad Sci U S A. 82:7314-7318.

Corvera,S., Whitehead,R.E., Mottola,C. and Czech,M.P. (1986). The insulin-like growth factor II receptor is phosphorylated by a tyrosine kinase in adipocyte plasma membranes. J. Biol. Chem. **261:** 7675-7679.

Corvera,S., Folander,K., Clairmont,K.B. and Czech,M.P. (1988). A highly phosphorylated subpopulation of insulin-like growth factor II/ mannose 6-phosphate receptors is concentrated in a clathrin-enriched plasma membrane fraction. Proc.Natl.Acad.Sci. USA. 85: 7567-7571.

Dahms,N.M., Lobel,P., Breitmeyer,J., Chirgwin,J.M. 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,N.M and Kornfled,S (1989). The cation-dependent mannose 6-phosphate receptor. J.Biol.Chem. **264:** 11458-11467.

Dahms,N.M., Lobel,P. and Kornfeld,S. (1989). Mannose 6-phosphate receptors and lysosomal enzyme targeting. J:Biol.Chem. **264:** 12115-12118.

Dahms,N.M., Rose,P.A., Molkentin,J.D., Zhang,Y. and Brzycki,M.A. (1993). The bovine mannose 6-phosphate/insulin-like growth factor 11 receptor. J.Biol.Chem. **268:** 5457-5463.

Dahms,N.M., Brycki-Wessell, M.A., Ramanujam, K.S. and Seetharam, B. (1993) Characterization of mannose 6-phosphate receptors (MPRs) from opossum liver: opossum cation-independent MPR binds insulin-like growth factor-II. Endocrionology **133:** 440-446.

Dahms,N.M., Wick,D.A. and Brzycki-Wessell,M.A. (1994). The bovine mannose 6-phosphate/insuline 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.

Dahms,N.M. and Hancock, M.K. (2002). P-type lectins. Biochem. Biophys. Acta. 1572:317-340.

Danser,A.H., Saris,J.J., Schuijt,M.P. and van Kats,J.P. (1999). Is there a local renin-angiotensin system in the heart? Cardiovasc. Res. **44:** 252-265.

David,L. Roberts., Daniel,J.Weix., Dahms, N.M. and Jung-Ja P.Kim. (1998). Molecular basis of lysosomal enzyme recognition : three dimensional structure of the cation-dependent mannose 6-phosphate receptor. Cell. **93:** 639-648.

De Souza, A.T., Hankins, G.R., Washington, M.K., Fine, R.L., Orton, T.C. and Jirtle, R.L. (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.

De **Souza,A.T.,** Hankins, G.R., Washington, M.K., Orton, T.C. and Jirtle, R.L. (1995). M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. Nat Genet. 11: 447-449.

Dell'Angelica,C.E., Puertollano,R., Mullins,C, Aguilar,C.R., Vargas,D.S., Hartnell,L.M. and Bonifacino,S.J. (2000). GGAs: A Family of ADP Ribosylation Factor-binding proteins Related to Adaptors and Associated with the Golgi Complex. J.Cell Biol. **149:** 81-94.

Dennis,P.A. and Rifkin,D.B. (1991). Cellular activation of latent transforming growth factor - beta 1 requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. Proc.Natl.Acad.Sci. USA. 88: 580-584.

Denzer,K., Weber,B., Hille-Rehfeld,A., von Figura,K. 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.

Devi,G.R., Byrd,J.C, Slentz,D.H. and MacDonald,R.G. (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. Endocrinology. 12: 1661-1672.

Devi,G.R., De Souza, A.T., Byrd, J.C., Jirtle, R.L. and MacDonaldR.G. (1999). Altered ligand binding by insulin-like growth factor II/mannose 6-phosphate receptors bearing misssense mutations in human cancers. Cancer Res. 59: 4314-4319.

Diaz,E. and Pfeffer,S.R., (1998). TIP 47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell. **93:** 433-443.

Dickson,R.B., Beguinot,L., Hanover,J.A., Richert,N.D., Willingham,M.C. and Pastan,I. (1983). Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc.Natl.Acad.Sci. USA. **80:** 5335-5339.

Distler,J.J. and George W, Jourdian. (1987). Low molecular weight phosphomannosyl receptor from bovine testes. Meth. Enzymol. **138:** 504-508.

Distler,J.J., Guo,J., Jourdian,G.W., Srivastava,O.P. 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.

Drickamer,K. and Taylor,M.E. (1993). Biology of animal lectins. Ann. Rev. Cell Biol. 9: 237-264.

Dubois,M.K.A., Gilles,J.K., Hamilton, P.A., Robbers and Smith,F. (1956). Colorimetric method for determination of sugars and related substances. Anal.Chem. **28:** 350-356.

Edman,P. and Begg,G. (1967). A protein sequenator.. Eur.J.Biochem. 1: 80-91.

Faust,P.L., Chirgwin,J.M. and Kornfeld,S. (1987). Renin, a secretory glycoprotein, acquires phosphomannosyl residues. J.Cell Biol. **105:** 1947-1955.

Fischer,H.D., Creek,K.E. and Sly, W.S. (1982). Binding of oligosaccharides to immobilized phosphomannosyl receptors. J.Biol.Chem. 257: 9938-9943.

Fraker,P.J. and Speck,J.C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, l,3,4,6-tetracholro-3a,6a-diphrenylglycouril. Biochem. Biophys. Res. Commun. **80:** 849-57.

Frenz,J., Quan,C.P., 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:** 335-400.

Gabel,C.A., Goldberg,D.E. 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.USA. **80:** 775-779.

Gabel,C.A., Dubey,L., Steinberg,S.P., Sherman,D., Gershon,M.D. and GershonA.A. (1989). Varicella-zoster virus glycoprotein oligosaccharides are phosphorylated during posttranslational maturation. J.Virol. **63:** 4264-4276.

Garmroudi,F. and MacDonald,R.G. (1994). Localization of the insulin-like growth factor II (IGF-II) binding/cross-linking site of the IGF-II/mannose 6-phosphate receptor to extracellular repeats. J. Biol. Che. **269:** 26944-26952.

Garmroudi,F., Devi,G., Slentz,D.H., Schaffer,B.S. and MacDonald,R.G. (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. Endocrionol. **10:** 642-651.

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.

Ghahary,A., Tredget,E.E., Shen,Q., Kilani,R.T., Scott,P.G. 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 derma fibroblasts. Growth factors. **17:** 167-176.

Glickman,J.N., Conibear,E. and Pearse,B.M.F. (1989). Specificity of binding of clathrin adaptors to signals on the mannose 6-phosphate /insulin-like growth factor II receptor. EMBO.J. 4: 1041-1047.

Glimore,R., Blobel, G. and Walter, P. (1982). Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J.Cell Biol. 95: 463-469.

Godar,S., Horejsi,V., Weidle,U.H., Binder,B.R., Hansmann,C. and Stockinger,H. (1999). M6P/1GF-II receptor complexes urokinase receptor and plasminogen for activationof transforming growth factor - beta 1. Eur. J. Immunol. 29: 1004-1013.

Goldberg,D.E., Gable,C.A. and Kornfeld,S. (1983). Studies of the biosynthesis of the mannose 6-phosphate receptor in receptor-positive and deficient cell lines. J.Cell Biol. 97: 1700-1706.

Gonzalez-Noriega,A., Grubb,J.H., Talkad,V. and Sly,W.S. (1980). Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J.Cell.Biol. **85:** 839-852.

Griffiths,G.M. 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.

Grimme,S., Honing,S., von Figura,K. and Schmidt,B. (2000). Endocytosis of insulin-like growth factor II by a mini-receptor based on repeat 11 of the mannose 6-phosphate/insulin-like growth factor II receptor. J. Biol. Chem. **275:** 33697-33703.

Groskopf,J.C., Syu,L.J., Saltiel,A.R. and Linzer,D.I. (1997). Proliferin induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase-dependent pathway. Endocrinology. **138:** 2835-2840.

Guarnieri,F.G., Arterbuen,L.M., Penno,M.B., Cha,Y. and August,J.T. (1993). The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. J.Biol.Chem. **268:** 1941-1946.

Hankins,G.R., De,Souza,A.T., Bentley,R.C, Patel,M.R., Marks,J.R., Igelhart,J.D. and Jirtle,R.L. (1996). M6P / IGF 2 receptor: a candidate breast tumor suppressor gene. Oncogene. 12: 2003-2009.

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

Hasilik,A. and Neufeld, E.F. (1980). Biosynthesis of lysosomal enzymes in fibroblasts. Phosphorylation of mannose residues J.Biol.Chem. 255: 4946-4950.

Hasilik,A., Waheed,A. and von Figura,K. (1981). Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine. Absence of the activity in I-cell fibroblasts. Biochem.Biophys.Res.Comm. 98: 761-767.

Hemer,F., Korner,C. and Braulke,T. (1993). Phosphorylation of the human 46kDa 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.

Heute-Perez,J.A., Engel,J.C., Brinen,L.S., Mottram,J.C. and McKerrow,J.H. (1999). Protease trafficking in two primitive eukaryotes is mediated by a prodomain protein motif. J.Biol.Chem. **274:** 16249-16256.

Hickman,S. and Neufeld,E.F: (1972). A hypothesis for I-cell disease: Defective hydrolases that do not enter lysosomes. Biochem.Biophys.Res.Comm. **49:** 992-999.

Hickman,S., Shapiro,L.J. and Neufeld,E.F. (1974). A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. Biochem.Biophys.Res.Commun. 57: 55-61.

Hieber,V., Distler,J., Myerowitz,R., Schmickel,R.D. and Jourdian,G.W. (1976). The role of glycosidically bound mannose in the assimilation of β-galactosidase by generalized gangliosidosis fibrobalsts. Biochem.Biphys.Res.Commun. 73: 710-717.

Hille,A., Waheed, A. and von Figura, K. (1989). The ligand-binding conformation of Mr 46000 mannose 6-phosphate receptor. Acquisition of binding activity during *in vitro* synthesis. J.Biol.Chem. **264:** 13460-13467.

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

Hirst,J., Lui,W.W.Y., Bright,N.A., Totty,N., Seaman,M.N.J. and Robinson,M.S. (2000). A Family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the *trans*-Golgi Network and the vacuole/Lysosome. J.Cell Biol. 149:67-80.

Hoflack,B. and Kornfeld,S. (1985a). Purification and characterization of a cation - dependent mannose 6-phosphate receptor from murine P388D| macrophages and bovine liver. J.Biol.Chem. 260:12008-12014.

Hoflack,B and Kornfeld,S. (1985 b). Lysosomal enzyme binding to mouse $P388D_1$ macrophage membranes lacking the 215 kDa mannose 6-phosphate receptor. Evidence for the existence of a second mannose 6-phosphate receptor. Proc.Natl.Acad.Sci. USA. 82: 4428-4432.

Hoflack,B., Fujimoto, K. and Kornfeld, S. (1987) The interaction of phosphorylated oligosaccharides and lysosomal enzymes with bovine liver cation-dependent mannose 6-phosphate receptor. J.Biol.Chem. **262:** 123-129.

Hoflack,B. and Lobel,P. (1993). Functions of the mannose 6-phosphate receptors. Advances in cell and Mol.Biol of membranes. 1: 51-80.

Honing,S. and Hunziker,W. (1995). Cytoplasmic determinants involved in direct lysosomal enzyme sorting, endocytosis and basolateral targeting of rat lpg 120 (Lamp I) in MDCK cells. J.Cell.Biol. **128:** 321-332.

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

Hynes,R. (1985). Molecular biology of fibronectin. Ann.Rev.Cell.Biol. 1: 67-90.

Ikushima,H., Munakata,Y., lshii,T., Iwata,S., Terashima,M., Tanaka,H., Schlossman,S.F. 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.

Jackson,D. and Linzer,D.I. (1997). Proliferin transport and binding in the mouse fetus. Endocrionology. **138:** 149-155.

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

Jeffrey Alan,M., David,A. Zopf and Victor Ginsburg. (1975). Affinity chromatography of carbohydrate specific immunoglobulins: coupling of oligosaccharides to Sepharose. Biochem.Biophys.Res.Comm. 62: 608-612.

Jirtle,R.L., Carr,B.L. and Scotl,C.D. (1991). Modulation of insulin-like growth factor-II/mannose 6-phosphate receptors and transforming growth facor-β 1 during liver regeneration. J.Biol.Chem. **266:** 22444-22450.

Jirtle,R.L., Haag,J.D., Ariazi,E.A. and Gould,M.N. (1993). Increased mannose 6-phosphate/insulin-like growth factor II receptor and transforming growth factor-β1 levels during monoterpene-induced regression of mammary tumors. Cancer Res. 53: 3849-3852

Johnson, F.K., Chan,W. and Kornfeld,S. (1990). Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. Proc.Natl.Acad.Sci. USA. 87: 10010-10014.

Johnson,F.K. 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,F.K. and Kornfeld,S. (1992b). The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor 11 receptor has two signals for lysosomal enzyme sorting in the Golgi. J.Cell.Biol. **119:** 249-257.

Kang,J.X., Li,Y. and Leaf,A. (1997). Mannose 6-phosphate /insulin-like growth factor II is a receptor for retinoic acid. Proc.Natl.Acad.Sci. USA. **95:** 13671-13676.

Kang,J.X., Bell,J., Leaf,A., Beard,R.L. and Chandraratna,R,A. (1998). Retinoic acid alters the intracellular trafficking of the mannose 6-phospahte/insulin-like growth factor II receptor and lysosomal enzymes. Proc.Natl.Acad.Sci. U.S.A. **95:** 13687-13691.

Kang,J.X., Bell,J., Beard,R.L. and Chandraratna,R.A. (1999). Mannose 6-phospahte/insulin-like growth factor II receptor mediates the growth-ingibitory effects of retinoids. Cell Growth Differ. 10:591-600.

Kaplan, A., Achord,D.T. and Sly,W.S. (1977). Phospho hexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proc.Nat.Acad.Sci. USA. 74: 2026-2030.

Keen, J.H., (1990). Clathrin and associated assembly and disassembly proteins. Ann.Rev.Biochem. **99:** 415-438.

Keinanen,K.P. (1988). Effect of deglycosylation on the structure and hormone-binding activity of the lutropin receptor. Biochem.J. **256:** 719-724.

Kessler,S.W. (1976). Use of protein A-bearing Staphylococci for the immunoprecipitation and isolation of antigens from cells. Meth. Enzymol. **73:** 442-59.

Kiely,M., McKnight, G.S. 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,M.M., Thomas,C.L., Nissley.S.P. and Sahagian,G.G., (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.

Killian,J.K. and Jirtle,R.L. (1999). Genome structure of the human M6P/IGF2 receptor. Mamm. Genome. 10: 74-77.

Killian,J.K., Buckley,T.R., Stewart,N., Munday,B.L. and Jirtle,R.L. (2001). Marsupials and Eutherians reunited: genetic evidence for the Theria hypothesis of mammalian evolution. Mamm. Genome. **12:** 513-517.

Klier,H.J., von Figura,K. and Pohlmann,R. (1991). Isolation and analysis of the human Mr 46000 mannose 6-phosphate receptor gene. Eur. J. Biochem. **197:** 23-28.

Klumperman,J., Hille-Rehfeld,A., Veenendal,T., Oorschot,V., Stoorvogel,W., von Figura,K. and Geuze,H.J. (1993). Differences in the endosomal distribution of the two mannose 6-phosphate receptors. J.Cell.Biol. **121:** 997-1010.

Kornfeld,S., Li,E. and Tabas,I. (1978). The synthesis of complex-type oligosachharides.II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein. J.Biol.Chem. **253:** 7771-7778.

Kornfeld,S. and Mellman,I. (1989). The biogenesis of lysosomes. Ann.Rev.Cell.Biol. 5: 307-330.

Kornfeld,S. (1992). Structure and function of the mannose 6-phosphate / insulin-like growth factor-II receptors. Ann.Rev. Biochem. **61:** 307-330.

Koster,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.

Kovacina,K.S., Steele-Perkins, G., Purchio,A.F., Lioubin, M., Miyazono,K., Heldin,CH. and Roth, R.A. (1989). Interactions of recombinant and platelet transforming growth factor-P 1 precursor with the insulin-like growth factor II/mannose 6-phosphate receptor. Biochem Biophys Res Commun. Apr **160:** 393-403

Kozak,M. (1981). Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acid Res. 9: 5233-5262.

Kozak,M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell. 44: 283-292.

Kuhn,C, Vielkind,U. and Anders,F. (1979). Cell cultures derived from embryos and melanoma of poeciliid fish. *In Vitro*. **15:** 537-544.

Kyle,J.W., Nolan,C.M., Oshima,A. and Sly,W.S. (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, U.K. (1970). Cleavage of structural proteins during the assembly of the head of T bacteriophage. Nature. **227:** 680-685.

Laureys,G., Barton,D.E., Ullrich,A. and Francke,U. (1988). Chromosomal mapping of the gene for the type-II insulin-like growth factor-II/cation-independent mannose 6-phosphate receptor in man and mouse. Genomics. 3: 224-229.

Lazzarino,D.A. and Gabel, C.A., (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., SchmidtA, Mauxion,F., Griffiths,G. and Hoflack,B. (1993). Binding of AP1 Golgi adaptors to membranes requires phosphorylated cytoplasmic domain of the mannose 6-phosphate/insulin-like growth factor II receptor. J.Biol.Chem. **268:** 22552-22556.

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,S.J. 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.

Li, E., Tabas,I. and Kornfeld,S. (1978). The synthesis of complex-type oligosaccharide-l. Structure of the lipid-linked oligosaccharide precursor of the complex-type oligosaccharides of the vesicular stomatitis virus G protein. J.Biol.Chem. **253:** 7762-7770.

Li,M., Distler,J.J. and Jourdian,G.W. (1990). The aggregation and dissociation properties of a low molecular weight mannose 6-phosphate receptor from bovine testes. Arch.Biochem.Biophys. **283:** 150-157.

Linnell,J., Groeger,G. and Hassan,A.B. (2001). Real time kinetics of insulin-like 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

Lippincott-Schwarz, J. and Fambrough,D.M. (1986). Lysosomal membrane dynamics: Structure and interorganellar movement of a major lysosomal membrane glycoprotein. J.Cell.Biol. **102:** 1593-1605.

Lobel,P., Dahms,N.M., Breitmeyer,J., Chirgwin,J.M. and Kornfeld,S. (1987). Cloning of the bovine 215 kDa cation-independent mannose 6-phosphate receptor. Proc.Natl.Acad.Sci. USA. **84:** 2233-2237.

Lobel,P., Dahms,N.M. 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,R.D., Griffiths,G. and Kornfeld,S. (1989). Mutations in the cytoplasmic domain of the 275 kDa mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. Cell. **57:** 787-796.

Lohi,O. and Lehto,V.P. (1998). VHS domain marks a group of proteins involved in endocytosis and vesicular trafficking. FEBS Lett. **440:** 255-257.

Lombardi,D., Soldati, T., Riederer, M.A., Goda, Y., Zerial,M. and Pfeffer,S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. EMBO J. **12:** 677-682.

Ludwig,T., Reuther,U., Metzger, R., Copeland,N.G., Jenkins, N.A., Lobel, P. and Hoflack,B. (1992). Gene and pseudogene of the mouse cation-independent mannose 6-phosphate receptor. J.Biol.Chem. **267:** 12211-12219.

Ma, Z.M., Grubb, J.H. and Sly, W.S. (1991). Cloning, sequencing and functional characterization of the murine 46 kDa mannose 6-phosphate receptor. J.Biol.Chem. **266:** 10589-10595.

Ma, Z.M., Grubb,J.H. and Sly,W.S. (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.

Mac.Donald,R.G., Pfeffer,S.R., Coussens,L., Tepper,M.A., Brocklebank.,C.M., Mole,J.E., Anderson,J.K., Chen,E., Czech,M.P. and Ullrich,A. (1988). A single receptor binds both insulin-like growth factor-II and mannose 6-phosphate. Science. **239:** 1134-1137.

Maomi,L., Jack,J.D. and George,W.J. (1989). Meth. Enzymol. **179:** 301-310.

Marron-Terada,P.G., Bollinger,K.E. and Dahms,N.M. (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,P.G., Mary,A.B.W. and Dahms,N.M. (1998). The two mannose 6-phosphate binding sites of the insulin-like growth factor -II / mannose 6-phosphate receptor display different ligand binding properties. J.Biol.Chem. 273: 22358-22366.

Marron-Terada,P.G., Hancock,M.K., Haskins,D.J. and Dahms,N.M. (2000). Recognition of *Dictyostelium discoideum* Lysosomal enzymes is conferred by the amino-terminal carbohydrate biding site of the Insulin-like growth factor-II/mannose 6-phosphate receptor. Biochemistry. **39:** 2243-2253.

Matzner,U., Annette,H.R., von Figura,K. and Pohlmann,R. (1996). Expression of mannose 6-phosphate receptors in chicken. Dev. Dynamics. **207:** 11-24.

McIntyre,G.F. and Erickson,A.H. (1993). The lysosomal proenzyme receptor that binds procathepsin L to microsomal membranes at pH 5 is a 43 kDa integral membrane protein. Proc.Natl.Acad.Sci.USA. **90:** 10588-10592.

Mehta,D.P., Ichikawa,M., Salimath,P.V., Etchison,J.R., Haak,R., Manzi,A. and Freeze,H.H. (1996). A lysosomal cysteine proteinase from *Dictyostelium discoideum* contains N-acetyl glucosamine-1-phosphate bound to serine but not mannose 6-phosphate on N-linked oligosaccharides. J.Biol.Chem. **271:** 10897-10903.

Mendez,E., Planas,J.V., 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.

Meresse,S., Ludwigm,T., Frank,R. and Hoflack,B. (1990). Phoshporylation of the cytoplasmic domain of the bovine cation-independent mannose 6-phosphate receptor. J.Biol.Chem. **265:** 18883-18842.

Meresse,S. and Hoflack,B. (1993). Phosphorylation of the cation-independent mannose 6-phosphate receptor is closely associated with its exit from the *trans*-Golgi Network. J Cell Biol. **120:** 67-75.

Merion,M. and Sly, W.S. (1983). The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. J. Cell Biol. **96:** 644-650.

Meyer,D.I., Krause, E. and Dobberstein, B. (1982). Title. Nature **297:** 647-650.

Miwako,I., Yamamoto, A., Kitamura,T., Nagayama,K. and Ohashi,M. (2001). Cholesterol requirement for cation-independent mannose 6-phosphate receptor exit from multivesicular late endosomes to the Golgi. J Cell Sci. 114: 1765-76.

Morgan,D.O., Edman,J.C, Standring,D.N., Fried,V.A., Smith, M.C., Roth, R.A. and Rutter, W.J. (1987). Insulin-like growth factor-II receptor as a multifunctional protein. Nature. **329:** 301-307.

Morris,S.A., Ahle, S. and Ungewickell, E. (1989). Clathrin-coated vesicles.. Curr. Opin. Cell Biol. 1: 684-690.

Motyka,B., Korbutt,G., Pinkoski,M.J., Heibein,J.A., Caputo,A., Hobman,M., Barry,M., Shostak,I., Sawchuk,T., Holmes,C.F., GauIdie,J. and Bleackley,R.C. (2000). Mannose 6- phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell induced apoptosis. Cell. **103:** 491-500.

Mullins,C. and Bonifacino, J.S. (2001). The Molecular machinery for lysosome biogenesis. BioEssays **23:** 333-343.

Munger,J.S., Harpel,J.G., Gleizes,P.E., Mazzieri,R., Nunes,I. and Rifkin,D.B. (1997). Latent transforming growth factor-beta structural features and mechanisms of activation. Kidney Int. **51:** 1376-1382.

Myerowitz,R. and Neufeld, E.F. (1981). Maturation of alpha-L-iduronidase in cultured human fibroblasts. J. Biol. Chem. **256:** 3044-3048.

Neufeld,E.F. and McKusick,V.A. (1983). Disorders of lysosomal enzyme synthesis and localization: I-cell disease and pseudo Hurler poly distrophy. In the metabolic basis of inherited disease, ed. J.B. Stanbury., J.B. Wyngaarden., D.S. Fredrickson., J.L. Goldstein., M.S. Brown. 778-787. New York: Mc Graw-Hill. 5th Edition.

Neufeld,E.F. (1991). Lysosomal storage diseases. Annu. Rev. Biochem. **60:** 257-80.

Nielsen,H., Engelbrecht,J., Brunak,S. and von Heijne,G. (1997). Identification of prokaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:** 1-6.

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.

Nunes,I., Shapiro,R.L. and Rifkin,D.B. (1995). Characterization of latent TGF-beta activation by murine peritoneal macrophages. J. Immunol. **155:** 1450-1459.

Nykjaer,A., Christensen,E.I., Vorum,H., Hager,H., Petersen,C.M., Roigaard,H., Min,H.Y., Vilhardt,F.,Moller,L.B., 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.

O'Dell,S.D. and Day,I.N. (1998). Insulin-like growth factor II (IGF-II). Int.J.Biochem. Cell Biol. **30:** 767-771.

Oeltmann,T., Carter,C., Merkle,R. and Moreman,K., (1994). The a-mannosidase of Trypanosoma cruzi: structure and function. Braz.J.Med.Biol.Res. 27: 483-488.

Ohkuma,S. and B.Poole (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl.Acad.Sci. USA. 75: 3327-3331.

Ohnuma,K., Munakata,Y., lshii,T., Iwata,S., Kobayashi,S., Hosono,O., Kawasaki,H., Dang,N.H. and Morimoto,C. (2001). Soluble CD26/dipeptidyl peptidase IV induces T cell proliferation through CD 86 upregulation on APCs. J.Immunol. **167:** 6745-6755.

Oka,Y., Rozek,L.M. and Czech,M.P. (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.

Orsel,J.G., Sincock,P.M., Krise,J.P. and Pfeffer,S.R. (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.

Oshima,A . Nolan,C.M., Kyle,J., Grubb,H.J. and Sly,W.S. (1988). The human cation-independent mannose 6-phosphate receptor. Cloning and sequencing of the full-length cDNA and expression of the functional receptor in COS-cells. J.Biol.Chem. **263:** 2553-2562.

Ouyang,H., Shiwaku,H.O., Hagiwara,H., Miura,K., Abe,T., Kato,Y., Ohtani,H., Shiiba,K., Souza,R.F., Meltzer,S.J. and Horii,A. (1997). The insulin-like growth

factor II receptor gene is mutated in genetically unstable cancers of the endometrium, stomach, and coleroctum. Cancer Res. 57:1851-1854.

Pearse,B.M.F. and Robinson,M.S. (1990). Clathrin adaptors and sorting. Ann. Rev.Cell.Biol. 6: 151-171.

Pelham,H.R.B. (1988). Evidence that luminal ER proteins are sorted from secreted proteins in post ER compartment. EMBO.J. 4: 2457-2460.

Perdue,J.F., Chan,J.K., Thibault,C, Radaj,P., Mills,B. and Daughaday,W.H. (1983). The biochemical characterization of detergent-solubilized insulin-like growth factor II receptors from rat placenta. J.Biol.Chem. **258:** 7800-7811.

Peters,C, Braun,M., Wendland,M., Schmidt,B., Pohlmann,R., Waheed,A. and von Figura,K. (1990). Targeting of lysosomal membrane protein: A tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for tartgeting to lysosomes. EMBO. J. 9: 3497-3506.

Pfeffer,S.R. (1988). Mannose 6-phosphate receptor and their role in targeting proteins to lysosomes. J. Membrane.Biol. **103:** 7-16.

Pohlmann,R., Waheed,A., Hasilik,A. and von Figura, K. (1982). Synthesis of phosphorylated recognition marker in lysosomal enzymes is located in the *cis* part of Golgi apparatus. J.Biol.Chem. **257:** 5323-5325.

Pohlmann,R., Nagel,G., Schmidt,B., Stein,M., Lorkowski,G., Krentler,C., Cully,J., Meyer,H.E., Grzeschik,K.H., Mersmann,G., Hasilik,A., and von Figura,K. (1987). Cloning od cDNA encoding the human cation-dependent mannose 6-phosphate specific receptor. Proc.Natl.Acad.Sci. USA. **84:** 5574-5579.

Pohlmann,R. (1996). Mannose 6-phosphate receptors. Biomembranes. **4:** 223-253.

Poussu,A., Lohi,O. and Lohto,V.P. (2000). Mmembrane transport structure function and biogenesis: Vear, a novel Glogi-associated protein with VHS and gama-adaptin "EAR" domain. J. Biol. Chem. **275:** 7176-7183.

Puertollano,R., Aguilar,R.C., Gorshkova,I., Crouch,R.J. and Bonifacino,J.S. **(2001).** Science. **292:** 1712-1716.

Purchio,A.F., Cooper,J.A., Brunner,A.M., Lioubin,M.N., Gentry,L.E., Kovacina,K.S., Roth,R.A. 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.

Radha,Y. and Siva Kumar,N. (2003). A new lactose specific lectin from the fresh water mussel *Unio*. Role of histidine residues in the biological activity of the lectin. Int. J. Bio-Chrom. **(In press).**

Reitman,M.L. and Kornfeld,S. (1981). Lysosomal enzyme targeting: N-acetyl glucosaminyl phosphotransferase selectively phosphorylates native lysosomal enzymes. J.Cell.Biol. 93:135-143.

Retzek,H., Steyrer,E., Sanders,E.J., Nimpf,J. and Schneider,W.J. (1992). Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. DNA Cell Biol. 11: 661-672.

Robey,P. and Neufeld, E.F. (1982). Defective phosphorylation and processing of beta-hexosaminidase by intact cultured fibroblasts from patients with mucolipidosis III. Arch. Biochem. Biopys. **213:** 251-257.

Robinson,M.S. (1992). Trends Cell Biol. 2: 293-297.

Rosorius,O., Mieskes,G., Issinger,O.G., Korner,C., Schmidt,B., von Figura,K. and Braulke,T. (1993). Characterization of phosphorylation sites in the cytoplasmic domain of the 300kDa mannose 6-phosphate receptor. Biochem. J. **292:** 833-838.

Roth,R.A., Stover,C., Hari,J., Morgan,D.O., Smith, M.C, Sara, V. and Fried,V.A. (1987). Interaction of the receptor for insulin-like growth factor II with mannose 6-phosphate and antibodies to the mannose 6-phosphate receptor. Biochem.Biophys.Res.Comm. **149:** 600-606.

Rothman ,J.E., Katz, F.N. and Lodish, H.F. (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,G.G., Distler,J. and Jourdian,G.W (1981). Characterization of a membrane associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular β-galactosidase. Proc.Natl.Acad.Sci.USA. 78: 4289-4293.

Sahagian,G.G. and Neufeld,E.F. (1983). Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. J.Biol.Chem. 258:7121-7128.

Sahagian,G.G. and Steer,C.J. (1985). Transmembrane orientation of the mannose 6-phosphate receptor in isolated clathrin-coated vesicles. J.Biol.Chem. **260:** 9838-9842.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989). Molecular cloning: A laboratory manual, 2 nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sando,G.N., Titus-Dillon,P., Hall,C.W. and Neufeld, E.F. (1979). Inhibition of receptor mediated uptake of a lysosomal enzyme into fibroblasts by chloroquine, procaine and ammonia. Exp.Cell.Res. **119:** 359-364.

Sanger,S.N. and Coulson,A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc.Natl.Acad.Sci. 74: 5463-5467.

Saris,J.J., Derkx,F.H., De Bruin,R.J., Dekkers,D.H., Lamers,J.M., Saxena, P.R., Schalekamp,M.A. and Jan Danser,A.H. (2001). High-affinity prorenin binding to cardiac mann-6-P/IGF-II receptors preceeds proteolytic activation to renin. Am.J.Physiol., Heart Circ.Physiol. **280:** H1706-H1715.

Saris,J.J., Derkx,F.H., Lamers,J.M. Saxena,P.R., Schalekamp,M.A. and Danser,A.H. (2001). 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 implication for the intracellular transport of thyroglobulin. Eur.J.Cell Biol. 49: 140-148.

Schmid,S.L. (1992). The mechanism of receptor-mediated endocytosis: more questions than answers. BioEssays. 14: 589-596.

Schmidt,B., Selmer,T., lngendoh,A. and von Figura,K. (1995). A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency. Cell: **82:** 271-278.

Schmidt,B., Kieke-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. (1997). Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail. PNAS. 94: 14471-14476

Shalamanova, L., Kubler, B., Jens-G, S. and Braulke, T., (2000) *Growth Hormone & IGF Research.* **10,** 294.

Siva Kumar.N. (1996). An affinity method for the purification of mannose 6-phosphatc receptor proteins (MPR 215) from rat tissues and goat liver. J.Biochem.Biophys.Methods. **31:** 181-184.

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.and Physiol. **118B**: 805-809.

Siva Kumar,N. and Udaya Lakshmi,Y. (1998). An efficient and rapid protocol for the purification and separation of mannose 6-phosphate receptor ptoreins (MPR 300 and MPR 46). Biochemistry, and Clinical Biochemistry. (Vol **12,** eds. T.C.Bog-Hansen and E.Van.Driessche)

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 teleostean fish (trout). Comparative Biochemistry and Physiology. **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.

Slodki, M.E. (1961). Phosphate linkages in phosphomannans from yeast. Biochim.Biophys.Acta. 57: 525-533.

Sly,W.S. and Fischer,H.D. (1982). The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. J.Cell:Biochem. 18:67-85.

Sosa,M.A., Schmidt,B., von Figura,K. and Hille-Rehfeld,A. (1993). *In vitro* binding of plasma membrane coated vesicle adaptors to the cytoplasmic domain of lysosomal acid phosphatase. J.Biol.Chem. **268:** 12537-12543.

Southern,E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J.Mol.Biol. **98:** 503.

Stein,M., Braulke,T., Krentler,C., Hasilik,A. and von Figura,K. (1987). 46 kDa mannose 6-phosphate specific receptor: purification, subunit composition, chemical modification. Biol.Chem. Hoppe-Seyler. **368:** 927-936.

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

Steiner,A.W. and L.H. Rome. (1982). Assay and purification of a solubilized membrane receptor that binds the lysosomal enzyme i -L-iduronidase. Arch.Biochem.Biophys. **214:** 681-687.

Stoger,R., Kubicka,P., Liu,C.G., Kafri,T., Razin,A., Cedar,H. and Barlow,D. (1993). Maternal-specific methylation of the imprinted mouse IGF 2r locus identifies the expressed locus as carrying the imprinting signal. Cell. 73: 61-71.

Stoorvogel,W., Geuze,H.J., Griffith,J.M., Schwartz,A.L. and Strous,G.J. (1989). Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein and mannose 6-phosphate receptor in human hepatoma cells. J.Cell.Biol. **108:** 2137-2148.

Suresh,K., Ramanadham, M. and Siva Kumar, N. (2002). An ELISA method to quantify mannose 6-phosphate receptors. J. Biochem. Biophys. Meth. **52:** 111-119.

Suresh,K. and Siva Kumar,N. (2003). An immno-affinity method for the purification of mannose 6-phosphate receptor protein. J. Biochem. Biophys. Meth. **(In press)**

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

Tabas,I. and Kornfeld,S. (1980). Biosynthetic intermediates of ، -glucoronidase contain high mannose oligosaccharides with blocked phosphate residues. J.Biol.Chem. **255:** 6633-6639.

Tong,P.Y., Tollefsen,S.E. and Kornfeld,S. (1988). The cation-independent mannose 6-phosphate receptor binds insulin-like growth factor II. J.Biol.Chem **263:** 2585-2588.

Tong,P.Y., Gregory,W. and Kornfeld, S. (1989). Ligand interactions of the cation-independent mannose 6-phosphate receptor. J.Biol.Chem. **264:** 7962-7969.

Tong,P.Y. and Kornfeld,S. (1989). Ligand interactions of the cation-dependent mannose 6-phosphate receptor. J.Biol.Chem. **264:** 7970-7975.

Trimble,R.B. and Maley, F. (1977). Sub-unit structure of structure of external invertase from *Sachharomyces cerevisiae.* J.Biol.Chem. **252:** 4409-4412.

Udaya Lakshmi,Y. and Siva Kumar,N. (1996). Affinity purification of mannose 6-phosphate receptor proteins: purification and partial characterization of goat liver receptors. Biochem, Mol. Biol.lnt. 40: 815-821.

Udaya Lakshmi,Y., Radha,Y., 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. Reports. **15:** 403-409.

Udaya Lakshmi,Y., Siva Kumar,N., Schu,P., von Figura,K. and Rehfeld,A.H. (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. (2000). Studies on mannose 6-phospahte receptors from non-mammalian vertebrates and invertebrates. Ph.D. Thesis.

Valdutiu,G.D. and Rattazzi, M.G. (1980). The effect of monensin on beta-hexosaminidase transport in normal and I-cell fibroblasts. Biochem. J. **192:** 813-820.

Van den Eijnden,M.M., Saris,J.J., de Bruin,R.J., De wit,E., Sluiter,W., Reudelhuber,T.L., Schalekamp,M.A., Derkx,F.H. and Danser,A.H. (2001). Prorenin accumulation and activation in human endothelial cells: importance of mannose 6-phosphate receptors. Arterioscler., Thromb., Vasc. Biol. **21:** 911-916.

Van Kesteren,C.A., Danser,AH. Derkx,F.H., Dekkers,D.H., Lamers,J.M. Saxena,P.R. and Schalekamp,M.A. (1997). Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cardiac cells. Hypertension. 30: 1389-1396.

Varki,A. and Kornfeld, S. (1980). Identification of a rat liver □-N-acetyl glucosaminyl phosphodiesterase capable of removing 'blocking' ! -N-acetyl glucosamine residues from phosphorylated high mannose oligosaccharides of lysosomal enzymes. J.Biol.Chem. **255:** 8398-8401.

Vidal,S., Garcia,M., Montero,J.L. and Morere,A. (2002). Synthesis and biological evolution of new mannose 6-phosphate analogues. Bioorganic & Medicinal Chem. 10: 4051-4056.

Villevalois-Cam,L., Rescan,C, Gilot,D., Ezan,F., Loyer,P., Desbuquois,B., Guguen-Guillouzo,C. and Baffet,G. (2003). The hepatocyte is adirect target for transforming-growth factor activation via the insulin-like growth factor II/mannose 6-phosphate receptor.. J. Hept. 38; 156-163.

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

von Figura,K. and Hasilik,A (1986). Lysosomal enzymes and their receptors. Ann.Rev.Biochem. **55:** 167-193.

Waheed,A., Hasilik,A. and von. Figura,K. (1981). Processing of the phosphorylated recognition marker in lysosomal enzymes. **256:** 5717-5721.

WaheedA-, Braulke,T., Junghans,U. and von Figura,K. (1988). Mannose 6-phosphate /insulin-like growth factor II receptor: The two types of ligands bind simultaneously to one receptor at different sites. Biochem.Biphys.Res.Comm. **152:** 1284-1254.

WaheedA, Gottschalk,S., HilleA, Krentler,C, Pohlmann,R., Braulke,T., Hauser,H., Geuze,H. and von Figura,K. (1988). Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. EMBO.J. 7: 2351-2358.

WaheedA and von Figura, K. (1990). Rapid equilibrium between monomeric, dimeric and tetrameric forms of the Mr 46000 mannose 6-phosphate receptor at 37°C. Possible relation to the function of the receptor. Eur.J.Biochem. **193:** 47-54.

WaheedA. HilleA, Junghans,U. and von Figura,K. (1990). Quaternary structure of the Mr 46000 mannose 6-phosphate specific receptor: effect of lignad, pH, and receptor concentration on equilibrium between dimeric and tetrameric receptor forms. Biochemistry. **29:** 2449-2455.

Wakamatsu,Y. (1981). Establishment of a cell line from the platyfish-swordtail hybrid melanoma. Cancer.Res. 41: 79-80.

Walter, P. and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature **299:** 691-698.

Wang,S., Souza,S.R.F., Kong,D., Yin,J., Smolinski,K.N., Zou,TX, Frank.T., Young,J., Flankers, K.C., Sugimura,H., Abraham,J.M. and Meltzer,S.J. (1997). Deficient transforming growth factor-p 1 activation and excessive insulin-like growth factor-II (IGF-I1) expression in IGF-II receptor mutant tumors. Cancer Res. 57: 2543-2546.

Wang,Z.H., Gershon,M.D., Lungu,O., Zhu,Z. and Gershon,A.A. (2000). Trafficking of varcella-zostr virus glycoprotein g1: T(338)-dependent retention in the trans-Golgi network, secretion, and mannose 6-phosphate-inhibitable uptake of the ectodomain. J.Virol. 74:6600-6613.

Wendland,M., Hille,A., Nagel,G., Waheed,A., von Figura,K. and Pohlmann,R. (1989). Synthesis of a truncated Mr.46000 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). The mutational analysis of disulfide bridges in the Mr 46000 mannose 6-phosphate receptor: localization and role for ligand binding. J.Biol.Chem. **206:** 7132-7136.

Wendland,M., Waheed,A., Schmidt,B., HilleA, Nagel,G., von Figura,K. and Pohlmann,R. (1991). Glycosylation of the Mr 46000 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:** 723-732.

Westcott,K.R. and Rome,L.H. (1988). The cation-independent mannose 6-phosphate receptor contains covalently bound fatty acid. J.Cell.Biochem. **38:** 23-33.

Westland,B., Dahms,N.M. 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.

Wiesmann,U.N., Di Donato,S. and Herschkowitz,N.N. (1975) Effect of chloroquine on cultured fibroblasts: release of lysosomal hydrolases and inhibition of their uptake. Bichem.Biophys.Res.Commun. **66:** 1338-1343.

Yamada,T., De Souza,A.T., Finkelstein,S. and Jirtle,R.L. (1997). Loss of the gene encoding mannose 6-phsopahte/insulin like growth factor-II receptor is an early event in liver carcinogenesis. PNAS. 94: 10351-10355.

Yandell,C.A., Dunbar,A.J., Wheldrake,J.F. and Upton,Z. (1999). The kangaroo cation-independent mannose 6-phosphate receptor binds insulin-like growth factor II with low affinity. J Biol Chem. 274: 27076-82.

Yunxiang,Z., Doray,B., Poussu,A., Lehto,V.P. and Kornfeld,S. (2001). Bindng of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor. Science **292:** 1716-1718.

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

Zhou,M., Ma,Z. and Sly,W.S. (1995). Cloning and expression of the cDNA of chicken cation-independent mannose 6-phosphate receptor. Proc.Natl.Acad.Sci. USA.**92**: 9762-9766.

Zhu,G., He,X., Zhai,P., Terzyan,S., Tang,J. and Xuejun,C. (2003). Crystal structure of GGA2 VHS domain and its implication in plasticity in the ligand binding pocket. FEBS Lett. **537:** 171-176.

PUBLICATIONS

1. **Suresh,K.,** Ramanadham, M. and Siva Kumar, N. (2002). An ELISA method to quantify mannose 6-phosphate receptors. J. Biochem. Biophys. Meth. **52: 111-119.**

2. **Suresh,K.** and Siva Kumar,N. (2003). An immno-affinity method **for** the purification of mannose 6-phosphate receptor protein. J. Biochem. Biophys. Meth. **(In press)**

ELSEVIER

J. Biochem. Biophys. Methods 52 (2002) 111–119

JOURNAL Of
biochemical and
biophysical
methods

www.elsevier.com/locate/jbbm

An ELISA method to quantify the mannose 6-phosphate receptors

Koduru Suresh, Madduri Ramanadham, Siva Kumar Nadimpalli *

Department of Biochemistry, University of Hyderabad, Hyderabad 500 046, India

Received 20 April 2002; accepted 6 June 2002

Abstract

Mannose 6-phosphate receptor proteins mediate transport of lysosomal enzymes to lysosomes in eukaryotes. Two receptors designated as MPR 300 and MPR 46 based on their apparent molecular mass have been well studied from human and bovine liver. In humans, it has been shown that the receptors are present in different concentrations in different tissues. In the present study, MPR 300 and MPR 46 were purified from goat liver by phosphomannan affinity chromatography, and polyclonal antibodies were raised in rabbits. MPR 300 receptor specific antibodies have been purified from the antiserum using a goat-MPR 300-receptor gel. Using this affinity-purified antibody and the antiserum to goat MPR 46, as well as an affinity-purified MSC1 antibody that is specific for MPR 46, we developed an ELISA method to quantify both the receptors. The receptors could be measured in the concentration range of 1–10 ng using ELISA. The receptors could be quantified from membrane extracts of different tissues of goat and chicken using this method.

Keywords: Mannose 6-phosphate receptor; F.LISA; Affinity purification

1. Introduction

Two mannose 6-phosphatc receptors that mediate transport of lysosomal enzymes to lysosomes in eukaryotes have been extensively characterized from human and bovine liver [1]. It has also been shown that the receptors transport distinct complements of lysosomal

Abbreviations: F.LISA, enzyme-linked immunosorbent assay; MPR 300 (Mr 300000), MPR 46 (Mr 46000) mannose 6-phosphate receptor.

* Corresponding author. Fax: +91-40-3010120.
E-mail address: nsksl@uohyd.ernet.in (S.K. Nadimpalli).

0165-022X/02/$ - see front matter
PII: SOI65-O22X(O2)OOO5O-7

enzymes. We purified both receptors from goat liver employing alternate affinity methods developed in our laboratory [2,3]. In order to understand the evolution of the receptor proteins, we have affinity-purified MPR 300 protein from the liver tissues of *Calotes versicolor* (reptiles), *Rana tigrina* (amphibians) and *trout* (pisces). MPR 46 could be weakly detectable from the liver tissues of reptiles and amphibians. Confirmatory evidence for the presence of MPR 46 among the amphibians (*Xenopus* oocytes) and fish (*Xiphophorus* cells) was obtained by metabolic labeling of the oocytes and fish cells. MPR 46 could be immunoprecipitated from these tissues by using an affinity-purified MPR 46 specific antibody (MSC1) raised against the synthetic peptide of the cytoplasmic tail of human MPR 46 protein. [4,5]. There has been a report on the development of an ELISA method to quantify the receptor proteins from different human tissues [6], and it has been shown that the levels of the receptors vary from tissue to tissue.

The objective of the present study was to develop a reliable and efficient ELISA method that could be useful to quantify the receptor proteins from membrane extracts of different species and different tissues of the same species, without purifying the receptor proteins. We have raised antibodies against purified goat MPR 300 and MPR 46 protein and also affinity-purified goat MPR 300 specific antibody on goat MPR 300 affigel. These antisera as well as the affinity-purified MSC1 antibody that is specific for MPR 46 and which has been shown to immunoprecipitate MPR 46 receptor from mammalian, and non-mammalian vertebrates, were used in the present study.

2. Materials and methods

2.1. Materials

ELISA plates (96 wells) were purchased from Greiner. Affigel-10 was purchased from Bio-Rad laboratories, USA. Imidazole, *p*-nitrophenyl phosphate, manganese chloride, Triton X-100, Sepharose 4B, divinyl sulfone, mannose 6-phosphate, Acrylamide, Bisacrylamide and Fruend's complete adjuvant were purchased from Sigma, USA. All other chemicals and reagents used in the study were of high quality and purchased from reputed firms.

2.2. Collection of tissues

Fresh liver, heart, spleen, brain, kidney from goat and chicken were collected on ice from the local animal suppliers and stored frozen at - 80 "C.

2.3. Preparation of the affinity matrix Sepharose-divinyl sulfone-phosphomannan and purifying the MPR 300 and MPR 46 protein

O-Phosphonomannan was a generous gift from Dr. M.E. Slodki, USA. It was hydrolyzed with acid and separated into phosphomannan core and pentamannosylphosphate. The affinity absorbent containing phosphomannan core was prepared in 20 ml batches as described earlier [3]. Acetone powder of goat and chicken liver tissue was prepared as

described [7| and total membrane proteins were extracted from the acetone powder and both MPR proteins were purificd from this by phosphomannan affinity chromatography in presence of divalent metal ions [5].

2.4. Raising antibodies to the purified goat liver MPR 300 and MPR 46 proteins

The homogeneity of the MPR proteins was assessed by 10% SDS-PAGE and silver staining. Purified receptors were extensively dialyzed against column buffer (50 mM imidazole–HCl buffer pH 7.0, 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05% Triton X-100, 2 mM EDTA) and reapplied onto phosphomannan Sepharose gel equilibrated with column buffer. The unbound fraction from the gel containing the MPR 46 was pooled and concentrated and the bound MPR 300 was specifically eluted from the gel using 5 mM mannose 6-phosphate in column buffer. Presence of MPR 46 alone in the unbound fraction and MPR 300 alone in the eluted fraction was confirmed by SDS-PAGE analysis of both fractions under reducing conditions and detected by silver staining [8]. Purified receptor (either MPR 300 or MPR 46) of 200 ug was acetone precipitated and thc pellet suspended in 500 ul of PBS. Five hundred microliters of Freund's complete adjuvant was added and the suspension subcutaneously injected into two separate rabbits. The rabbits received booster doses at third and fifth week. The rabbits were bled by ear vein puncture after which the scrum was collected and kept frozen at - 20 °C. Affinity-purified MSC1 antibody (this antibody was raised against a synthetic peptidc of the cytoplasmic tail of the human liver MPR 46 protein and was affinity-purified on thc immobilized peptide gel) is highly specific for MPR 46 protein and was generously provided by Prof. Dr. K. von Figura, Goettingen, Germany.

2.5. Affinity purification of anti-receptor antibodies on receptor affinity gel

Goat MPR 300 protein purified on phosphomannan gel was concentrated by Amicon concentrator (PM 10 membrane) and 1 mg of the receptor was coupled to 1.0 ml of Affigel-10 in presence of 5 mM mannose 6-phosphate employing the conditions described by the manufacturer. Antiserum to purified goat MPR 300 protein was extensively dialyzed against 10 mM Tris–HCl buffer pH 7.4 containing 150 mM sodium chloride (column buffer) and then applied to the receptor affigel at 4 °C equilibrated with same buffer. After extensively washing the gel with column buffer, bound lgG was specifically eluted with three column volumes of 100 mM glycine- HC1 buffer pH 2.65. The eluted protein was immediately neutralized with 2 M Tris, analyzed on a 7.5% SDS-PAGE under non-reducing conditions and stored at 4 °C. From 10 ml of antiserum, 100 ug of purified IgG was obtained.

2.6. Western blot analysis

To detect the specificity of the antibodies, goat liver MPR 300 protein and MPR 46 as well as the chicken liver MPR 300 and MPR 46 that were affinity-purified on phosphomannan gel were separated on a 10% SDS-PAGE and the proteins were trans-

ferred to a nitrocellulose membrane. The membrane was incubated with affinity-purified goat MPR 300 IgG (10 ug) to detect goat and chicken MPR 300 proteins. To detect the goal and chicken MPR 46 proteins, antiserum to purified goat MPR 46 protein (1:1000 dilution) and MSC1 antibody (8 ug) were used as the primary antibodies. The receptor bands were finally detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate (Bangalore Genei, Bangalore).

2.7. Extraction of membrane proteins from the tissues

One gram of each tissue was processed to get the membrane proteins as follows [6]. An amount 1 ml of 100 mM sodium acetate buffer pH 6.0 containing 0.2 M NaCl, 5 mM iodoacetamide, 1 mM EDTA and 1 mM PMSF was added to 1 g of the tissue that has been minced well. The suspension was thoroughly homogenized and centrifuged for 40 min at 40000 rpm in an ultracentrifuge and the soluble extract was discarded. The pellet was then extracted with 50 mM imidazole HCl buffer pH 7.0 containing protease inhibitor (1 mM PMSF), 1 mM EDTA and 0.5% Triton X-100 and incubated on ice for 15 min. The suspension was re-centrifuged in an ultracentrifuge as above to get the clear membrane extract. The pellet was re-extracted once to obtain membrane proteins. Protein concentration in the combined extracts was determined by Peterson's [9] method using BSA as standard.

2.8. ELISA method for the quantification of the purified receptors

Affinity-purified antibodies against MPR proteins were adsorbed to microtiter wells of an ELISA plate (96 wells) for 4 h at 37 °C (250 ng of affinity-purified goat MPR 300 antibody or 1 ug of affinity-purified MSC1 antibody, in 50 ul of 25 mM Tris–HCl, pH 7.0). The wells were washed with 200 ul of 25 mM Tris-HCI buffer pH 7.0 and incubated overnight at 4 °C/1 h at room temperature with 200 ul of buffer C (5% lipid-free milk powder. 0.05% Triton X-100, 10 mM sodium phosphate, 150 mM NaCl pH 7.4). Fifty microliters of receptor-containing solution (0.5-10 ng) of purified MPR 300 or MPR 46 from goat liver, or different tissue extracts of goat and chicken (0.5 5.0 ug concentration diluted in Buffer C) (quantitation of protein was done according to Peterson), was bound for 2.5 h at 37 °C. The wells were washed four times with 200 ul of buffer D (0.05% Triton X-100, 10 mM sodium phosphate, 150 mM NaCl pH 7.4) followed by incubation with 200 ul of buffer C for 30 min at 37 °C. Subsequently, 50 ul of diluted rabbit antiserum against M6P receptors (dilution in buffer C, 10^{-3} for MPR 300 antiserum or 10^{-2} for MPR 46 antiserum) was added and the plate incubated for 1 h at 37 °C. After washing four times with buffer D, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:2000 in buffer C) was added and incubated for 1 h at 37 °C. The wells were washed four times with buffer D and one time with 200 ul of buffer E (0.1 M Tris-HCI, 0.1 M NaCl, 2 mM $MgCl_2$, pH 9.5). The colour was developed with *p*-nitrophenyl phosphate (1 mg/ml in buffer E) for 10 to 20 min at room temperature and the absorbance was measured at 405 nm in a microplate ELISA reader.

3. Results and discussion

When the total membrane extract from goat liver as well as chicken liver was passed through separate phosphomannan Sepharose gels and the bound proteins eluted specifically with 5 mM mannose 6-phosphate and analyzed on SDS-PAGE consistent with our earlier findings, both MPR 300 and MPR 46 protein bands could be detected from both species. Both proteins could be completely separated from one another by passing the mixture on phosphomannan Sepharose gel in presence of 2 mM EDTA as described under Materials and methods. Eluted and unbound fractions showed MPR 300 and MPR 46, respectively, in both species (Fig. 1). Antiserum to the purified goat MPR 300 and MPR 46 receptors was separately raised in two rabbits as described under Materials and methods.

Our earlier studies established that an antibody to the goat MPR 300 protein cross-reacts with the receptor from the chicken and reptiles [4]. Affinity-purified MSC1 antibody is highly specific for MPR 46 and has been shown to recognize the MPR 46 receptors from goat and different non-mammalian vertebrates [4]. In the present study, an antibody was raised for the purified MPR 46 protein from the goat liver.

The authenticity as well as the specificity of both proteins was further confirmed by Western blot studies. From Fig. 2a, it is clear that the purified goat and chicken liver MPR 300 protein react specifically with an affinity-purified goat MPR 300 antibody. MPR 46 from both species could also be detected specifically using an antiserum to goat MPR 46 protein as well as by the affinity-purified anti-MSC1 antibody (Fig. 2b). These different receptor specific antibodies were used to develop an ELISA method to quantify MPR 300 and 46 receptors.

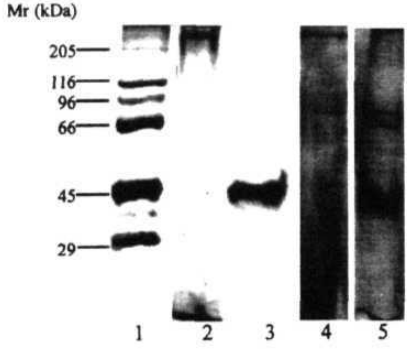


Fig. 1. 10% SDS-PAGE under reducing conditions of purified MPR proteins from goat (lanes 2 and 3) and chicken (lanes 4 and 5). Lane 1 standard markers, lanes 2 and 4 MPR 300 (aliquot of eluted sample) and lanes 3 and 5 MPR 46 (aliquot of unbound sample).

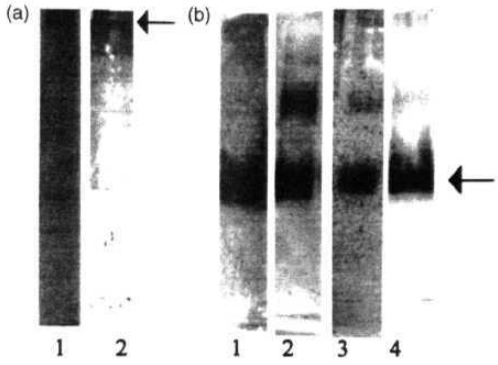


Fig. 2. Immuno blot analysis of purified receptors, Details are given in the text. (a| Lanes 1 and 2 represent goat and chicken MPR 300 proteins, respectively, reacted with affinity-purified anti-goat MPR 300 antibody. Arrow indicates position of MPR 300 protein. (b) Purified MPR 46 goat (lanes 1 and 2) and chicken (lanes 3 and 4). Lanes 1 and 3 treated with anti-goat MPR 46 antiserum and lanes 2 and 4 treated with affinity-purified MSC1 antibody. Arrow indicates position of MPR 46 protein.

In order to establish precisely the concentration of the receptor that could be detectable by the antibodies developed, in an EI.1SA method, initial ELISA was carried out employing purified goat MPR 300 and MPR 46 protein. From Fig. 3, it is clear that the

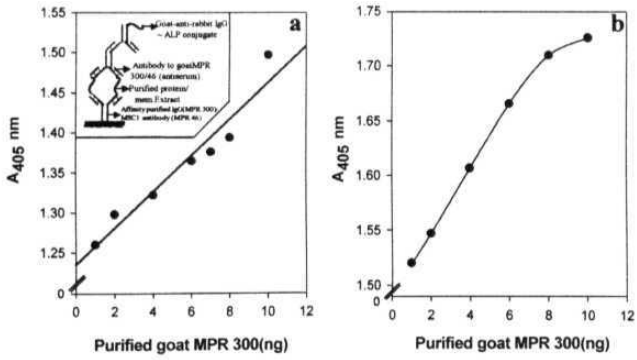


Fig. 3. Standard curve with purified goat MPR 300 (a) and MPR 46 (b). ELISA was carried out as described under the Materials and methods. A representative experiment, from a total of five, is shown.

level of detection of the MPR 300 and MPR 46 lies in the range of 1 - 10 ng. Using these standard graphs, the receptor concentration was quantified by ELISA employing the total membrane extract obtained from the liver tissue of goat (mammalian vertebrate) and chicken (non-mammalian vertebrate). With an increase in the protein concentration, there was a linear increase in the detectable range of MPR 300 and MPR 46 receptors for both species (Fig. 4).

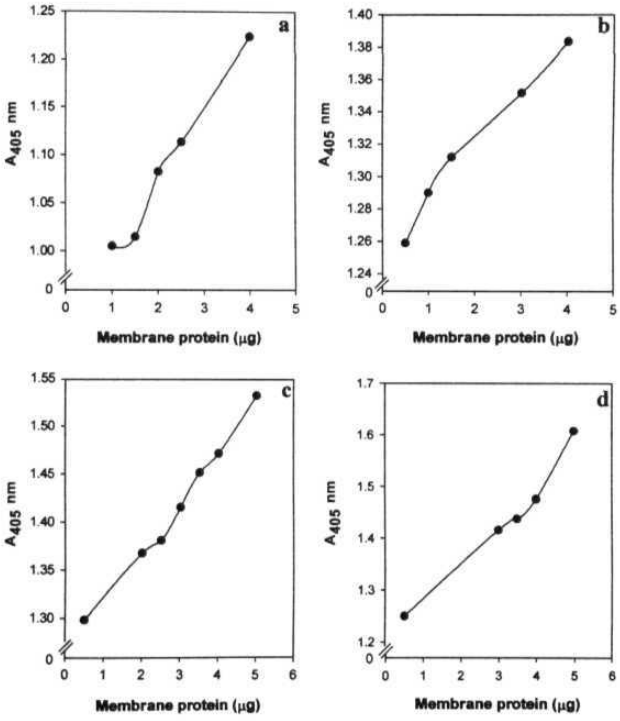


Fig. 4. ELISA to delect the MPR proteins in the liver membrane extracts of goat (a and b] and chicken (c and d). (a) and (c) represent MPR 300, (b) and (d) represent MPR 46. Fifty microliters of membrane extract at varied protein concentration was incubated with 250 ng of affinity-purified anti-goat MPR 300 antibody or with 1 ug of affinity-purified MSC`1 antibody. The assay was performed as described under Materials and methods. A representative experiment, from a total of four, is shown.

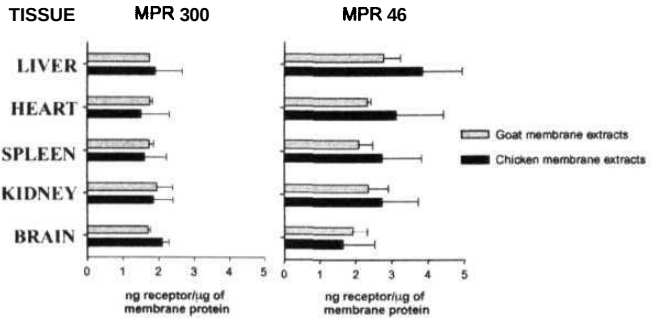


Fig. 5. Quantitation of MPR 300 and MPR 46 in different tissues of goat and chicken. ELISA was performed as described under Materials and methods. Data are average values of three independent experiments. The bars indicate standard deviations.

Having established the method developed which can be used to quantify the receptors in liver tissue, we analyzed the levels of both receptor proteins from other different tissues (heart, spleen, kidney and brain) of goat and chicken. Results of these experiments are shown in Fig. 5.

Comparison of the levels of MPR 300 and MPR 46 in different tissues of goat and chicken indicates that the levels of MPR 300 and MPR 46 are variable. However, the levels of MPR 46 among different tissues of chicken are higher than those found in goat.

The results obtained in the present study clearly demonstrate the specificity of the receptor specific antibodies from a mammalian species to detect putative homologous receptor proteins among different vertebrate species. Due to the high sensitivity of the assay microgram amount of tissue proteins is sufficient for the quantification of the MPR proteins. Further, the solid phase assay allows us to process many samples at a time. The data allow us to compare the levels of both receptors in different tissues of goat and chicken. Our earlier studies have already confirmed that both MPR 300 and MPR 46 receptors are consistently present among all vertebrates including fish, with biochemical and immunological properties similar to the goat receptors [5]. Using the ELISA method developed in the present study, it is possible to quantify the receptors from different vertebrates and also allows us to compare the steady state concentration of the two receptors in different tissues of the same animal species or different species, which have so far been shown to contain both receptors. The method can be well extrapolated to quantify the receptors in different animal species at different stages of growth and development.

Acknowledgements

The authors are grateful and thankful to the Department of Science and Technology, New Delhi for the research support given for this work in the form of a Project: SP/SO/

D21/97. K. Suresh is a Research Fellow in the Project. The authors arc grateful to Prof. Dr. K. von Figura for kindly providing the affinity-purified MSCl antibody used in this study.

References

[1] Hill-Rehfeld A. Biochem Biophys Acta 1995;1241:177 94.
[2] Siva Kumar N. J Biochem Biophys Methods 1996;31:181 4.
[3] Udaya Lakshmi Y. Siva Kumar N. Biochem Mol Biol Int 1996;40:815 21.
[4] Siva Kumar N. Hille-Rehfeld A. von Figura K. Comp Biochem Physiol 1997;118B:805 9.
[5] Siva Kumar N. Udaya Lakshmi Y, Hille-Rehfeld A, von Figura K. Cotnp Biochem Physiol 1999;123B:261 - 5.
[6] Wenk J. Hille A. von Figura K. Biochem Int 1991;23:723- 32.
[7] Distler JJ, Jourdian GW. Methods Enzymol 1989;138:5O4- 8.
[8] Ansorge W. J Biochem Biophys Methods 1985:11:13- 20.
[9] Peterson GL. Anal Biochem 1977;83:346–56.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
J. Biochem. Biophys. Methods xx (2003) xxx–xxx
JOURNAL OF biochemical and biophysical methods
www.elsevier.com/locate/jbbm

An immuno-affinity method for the purification of mannose 6-phosphate receptor proteins

Koduru Suresh, Siva Kumar Nadimpalli *

Biochemistry Laboratory, Department of Biochemistry, University of Hyderabad, Hyderabad 500 046, India

Received 17 June 2003; accepted 30 June 2003

Abstract

In a recent study, we have developed an ELISA method to quantify the mannose 6-phosphate receptor (MPR) proteins [J. Biochem. Biophys. Methods 52 (2002) 111]. In the present study, we have used the goat MPR 300 antibody and peptide specific antibodies to human MPR 46 to develop simple and **efficient** immuno-affinity matrices, which can be used to purify the MPR proteins from goat liver in a single step. The **identity** of the immuno-affinity purified receptors is confirmed by their molecular masses as well as by their immunoreactivity.

Keywords: MPR **proteins;** Immuno-affinity; Peptide-specific antibodies

1. Introduction

Mannose 6-phosphate receptor proteins mediate transport of lysosomal enzymes in eukaryotes. Two receptors designated as MPR 300 and MPR 46 based on their apparent molecular masses have been extensively characterized in mammals [1]. Receptors with similar molecular mass and biochemical and immunological characteristics have also been identified from **different** non-mammalian vertebrates such as fish, amphibians, reptiles and birds [2]. **Further** a partial cDNA clone isolated for the fish MPR 300 protein revealed extensive sequence homologies among the different vertebrate MPR 300 proteins [3]. Recently, the MPR's have also been identified from the invertebrate molluscs [4]. Though extensive work has been carried out on these

* Corresponding author. Tel.: +91-40-23010500; fax: +91-40-23010120.
E-mail address: nsksl@uohyd.ernet.in (S.K. Nadimpalli).

0165-022X/03/$ - see front matter
doi:10.1016/S0165-022X(03)00148-9

receptors from mammals, very little is known about their functions in other animal species. There has been no published information on the immuno-affinity purification of these receptors. Our laboratory has raised polyclonal antibodies to the purified goat receptors. Additionally, peptide specific antibodies were raised for distinct peptide sequences of the cytoplasmic tail of the human MPR 46 protein. In the present paper, we describe the development of a simple immuno-affinity method for the purification of the goat mannose 6-phosphate receptor proteins. In view of the similar biochemical and immunological properties exhibited by both the receptors among different animal species, this method should be useful to isolate and purify the receptor proteins in a single step from membrane extracts of animals that contain the receptors.

2. Materials and methods

2.1. Materials

Fresh goat liver tissue was obtained from the local slaughter house and transported on ice to the laboratory for use. *O*-phosphonomannan was a generous gift from Dr. M.E. Slodki, USDA, Peoria, IL, USA. Sepharose 4B, divinylsulfone, Freund's adjuvant (complete and incomplete), acrylamide, and bisacrylamide were purchased from Sigma, USA. Affigel-10 was purchased from BioRad Laboratories, USA. All other chemicals and reagents used in this study were of high quality and purchased from reputed firms locally.

2.2. Affinity purification of goat MPR 300 protein and raising antibodies

Sepharose–phosphomannan was prepared as described earlier and the goat MPR 300 protein was purified to homogeneity from liver membrane extracts. Antibodies were raised for the purified receptor in a rabbit. From the antiseruni, MPR 300 specific IgG was isolated on goat MPR 300-affigel as described [5].

2.3. Preparation of peptide specific antibody Jar the human MPR 46 protein

Peptides corresponding to residues 237–253 (peptide A) (ADGCDFVCRSKPRBVPA) and residues 259–276 (peptide B) (GDDZLGEESEERDDHLLP) of the cytoplasmic tail of human MPR 46 (numbering of the tail starts with Arg-212 [Pohlmann et al., PNAS 1988, 84J) were synthesized using F-moc-protected. PyBOP-(benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosponium hexafluorophosphate) activated amino acids on an automatic peptide synthesizer (Milligen 9050, Eschborn, Germany) and were kindly provided by Prof. Dr. K. von Figura (Goettingen, Germany). The peptide was purified by reverse-phase chromatography and conjugated to keyhole limpet hemocyanin using glutaraldehyde [6]. The conjugate (corresponding to 0.025 mg peptide in 500 µl) was mixed with equal volume of Freund's complete (first injection) or incomplete (booster injections) adjuvant and injected subcutaneously into rabbit. Booster injection was given 4 weeks after the first injection at biweekly intervals.

2.4. Preparation of the affinity gels containing goat MPR 300 and MPR 46 synthetic peptides

Affinity gel containing purified goat MPR 300 was prepared as described [5]. Peptides A and B were coupled to affigel-10 following manufacturer's instructions. Briefly. 2 ml of affigel-10 was extensively washed in a 10 ml sintered syringe with 7 to 10 bed volumes of chilled isopropanol. followed by water and 100 mM HEPES buffer pH 7.4. Finally to this, peptides A and B (10 mg/ml in 100 mM HEPES buffer pH 7.4) were added. Coupling was allowed to proceed for 24 h at 4 °C. The unbound fraction was collected, and the unreacted sites in the gel were blocked with 0.1 ml of 1 M ethanolamine–hydrochloride pH 8.0 for 1 h. Finally, the gel was washed extensively with PBS and stored at 4 °C in PBS until use. Four milligrams of 237 and 3.5 mg of 259 peptides per ml were coupled.

2.5. Isolation of peptide specific IgG (237 and 259) for MPR 46

Antisera to MPR 46 synthetic peptides were separately dialyzed against 10 mM Tris HCl buffer pH 7.4 containing 150 mM sodium chloride (column buffer) and then applied to the respective peptide-MPR 46 affigels at 4 °C equilibrated with the same buffer. After extensively washing the gel with the column buffer, bound IgG was specifically eluted with three column volumes of 100 mM glycine–HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2 M Tris. analyzed on a 7.5% SDS-PAGE under nonreducing conditions and stored at 4 °C. Yields of specific IgGs were comparable with our earlier findings [5].

2.6. Preparation of immuno-affinity gels

MPR 300 specific IgG (2 mg) as well as MPR 46 synthetic peptide specific IgG (5.5 mg of 237 and 2.0 mg of 259) were coupled separately to 1 ml of affigel-10 as described above.

2.7. Isolation of total membrane proteins from goat liver tissue

2.7.1. Immuno-affinity purification of goat MPR 300

All operations were performed at 4 °C. Total membrane proteins were obtained as described [7]. Fresh/frozen goat liver (100 g) was diced, and homogenized for 1 min with the help of a homogenizer in 200 ml of a solution containing 0.1 N acetic acid, and 0.1 M NaH_2PO_4. The homogenate was centrifuged for 15 min at 10,000 × *g*. The pellet was washed once with 350 ml of distilled water and the suspension recentrifuged at the same speed. The pellet was resuspended in 750 ml of imidazole–HCl buffer pH 7.0 containing 0.4 M KCl and 1% Triton X-100. The suspension was stirred for 60 min and then centrifuged for 60 min at 22.000 x *g* (Heraues, Biofuge Stratos, Germany). The pH of the supernatant was adjusted to 6.5 with 2 N acetic acid and immediately subjected to immuno-affinity chromatography on a column, equilibrated with imidazole HCl buffer pH 6.5, containing 150 mM NaCl, 0.05% Triton X-100 and 0.02% NaN_3 (column buffer). The gel was washed extensively with the column buffer. Finally, bound protein was eluted

with glycine–HCl buffer pH 2.65. Aliquot of the protein was TCA precipitated, and subjected to SDS-PAGE analysis and also for western blotting.

2.7.2. Immuno-affinity purification of the goat MPR 46 protein

For the purification of the goat MPR 46 protein, total membrane extracts were prepared from the acetone powder of the goat liver tissue as described [8], This was applied to the peptide affigels, equilibrated with (Tris–HCl buffer pH 8.0 containing 0.01% Triton X-100). The gels were extensively washed with the same buffer and bound protein was eluted using 100 mM glycine-HCI buffer pH. 2.65. The protein eluted was neutralized and analyzed by SDS-PAGE [9] and the MPR 46 receptor identified by silver staining [10]. In order to further characterize the binding of the goat MPR 46 protein to the peptide IgG gel, 2 ug of purified goat MPR 46 protein was iodinated with 100 uCi of $Na^{125}I$ using iodogen, as described to a specific activity of 1 x 10^7 cpm/ug [11].

2.8. Immunoprecipitation of MPR 46 using iodinated goat MPR 46 protein

Radiolabeled goat MPR 46 protein (50,000 cpm) was applied on both the peptide - affigel columns, equilibrated with Tris-HCl buffer pH. 7.4 containing 150 mM NaCl. After washing the gel with the same buffer, bound radioactivity could be specifically elutcd using 100 mM glycine-HCI butter pH 2.65. In a separate experiment. 50.000 cpm of the iodinated goat MPR 46 protein was incubated with different concentrations of affinity purified 237 peptide IgG (30–65 µg) in a total volume of 50 ul of PBS in 0.02% Tween 20. After an overnight incubation in cold, the samples were treated with 20 ul of pansorbin (Calbiochem). Pansorbin pellets were washed with PBS Tween and the specific radioactivity bound to the pellets were counted using a gamma-ray counter. Pellets were then cooked with SDS sample buffer and the clear supernatants subjected to 10% SDS-PAGE analysis. These studies were done only for 237 peptide specific antibody.

3. Results and discussion

Our long-term objective is to understand the structure and function of mannose 6-phosphate receptors from different animal species and to establish their evolutionary pattern. Towards achieving these goals, we have first identified mammalian homologous of MPR 300 and 46 among different nonmammalian vertebrates [3], and more recently established the appearance of both MPR proteins among the invertebrate molluscs [4]. In a recent study, it has been shown by us that the mannose 6-phosphate receptor proteins (MPR 300 and 46) from different animal species can be quantified using an ELISA method, employing antibodies to purified goat MPR 300 protein, and goat MPR 46 protein and an MSC1 antibody (that is specific to the cytoplasmic tail of the human MPR 46 protein) respectively [5].

In the present study, new methodology was developed for the purification of the goat MPR 300 and 46 proteins, using immuno-afTinity matrices. As a first step towards developing immuno-afTinity matrices for the purification of mannose 6-phosphale receptor proteins, polyclonal antibodies to the purified goat MPR 300 and peptide-specific anti-

bodies corresponding to the distinct regions of the cytoplasmic tail of the human MPR 46 protein were raised in rabbits.

From these antisera, MPR 300 specific IgG was purified on immobilized MPR 300 affigel. From 10 ml of antiserum, 100 μg of the specific IgG could be obtained. Fig. 1 shows the migration of specific IgG on 7.5% denaturing gel in the absence of reducing agents. From 7 ml of the peptide specific antiserum. 1.5 mg of 237 IgG was obtained and from 10 ml of the peptide specific antiserum 1.35 mg of 259 IgG could be obtained. Additional quantities of peptide specific IgG's were obtained by applying additional antisera on the respective peptide- affigels.

Purified MPR 300 specific IgG could be coupled to affigel at a concentration of 1 mg/ml, and the peptide specific IgGs at a concentration of 5.5 mg and 2.0 mg per ml of affigel. When the membrane extracts of goat liver were passed through the MPR 300 IgG gel, as described under materials and methods the receptor bound to the gel and could be specifically eluted using 100 mM glycine HCl buffer pH 2.65. Protein (140 ug) was obtained from 100 g of tissue. Fig. 2A shows the SDS-PAGE of the receptor. (The receptor purified on phosphomannan–sepharose gel served as control.) In order to confirm that this protein is indeed the putative goat MPR 300, an aliquot of the eluate was electrophoresed and subjected to western blot analysis using anti-goat MPR 300 antiserum. From Fig. 2B, it is apparent that the protein is the goat receptor.

When the total liver membrane extracts from goat were applied on either of the peptide gels, and eluted with 100 mM glycine–HCl buffer pH 2.65, protein could be eluted from the gel and the 46 receptor band could be visualized on a 10% SDS-PAGE gel (data not shown). In order to further characterize the binding of the goat MPR 46 onto either of the

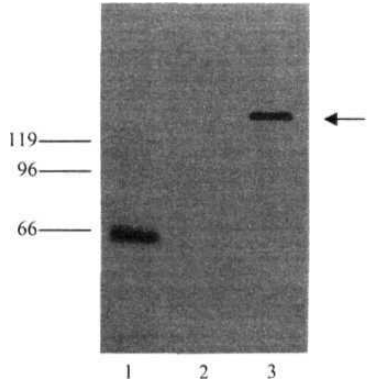
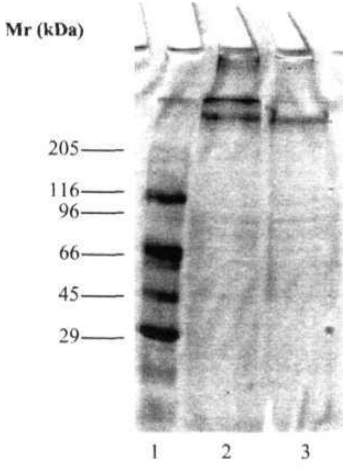
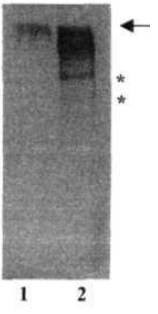


Fig. 1. 7.5% SDS-PAGE. analysis of the Goat MPR 300 IgG purified on goal receptor gel under nonreducing conditions; lane 1, standard molecular weight markers; lane 3. arrow indicates position of purified IgG.



A



B

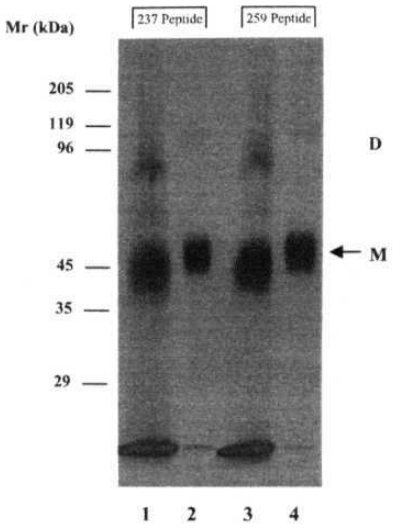


Fig. 3. 10% SDS-PAGE analysis of the iodinated goat MPR 4o on 2.17 and 259 peptide affigels (lanes 1 and 3 unbound fractions and lanes 2 and 4 elutions). M and 1) denote the monomer and SDS-resistant dimer of MPR 46.

affinity gels, the receptor eluted ffom the 237 peptide gel was concentrated and an aliquot of the same was acetone precipitated and radioiodinated.

When 50,000 cpm of the radioiodinated goat MPR 46 receptor was applied to 0.2 ml of the 237 and 259 IgG gels, 7000 cpm of the goat receptor could be specifically eluted from the gels using glycine–HCl buffer pH 2.65 (the counts could be completely TCA precipitated). A fraction of the **receptor** also appeared in the unbound fraction (Fig. 3). Additionally, the appearance of the SDS-resistant dimer of the goat receptor in addition to the monomer in the eluates of both gels confirms that the receptor bound to the gels.

In an additional experiment when 50,000 cpm of radioiodinated MPR 46 was incubated with different concentrations of the affinity purified 237 peptidc specific IgG (30-65 ng), binding of the receptor to the IgG was found to be linear (Fig. 4), supporting the specificity of recognition.

From the above data, it is evident that the immuno-affinity matrices can be used to purify the receptors from membrane extracts of the goat liver. It has earlier been shown

Fig. 2. (A) 10% SDS-PAGE analysis of MPR .100. Lane 1, standard molecular weight markers; lane 2. goal MPR 300 purified on immuno-affinity gel; lane 3. goat MPR 300 purified on PM gel (arrow indicates the position of receptor). (B) Immuno-blot analysis of goat MPR 300 purified on immuno-affinity gel (lane 1), purified on PM gel (lane 2) (•possible proteolytic products).

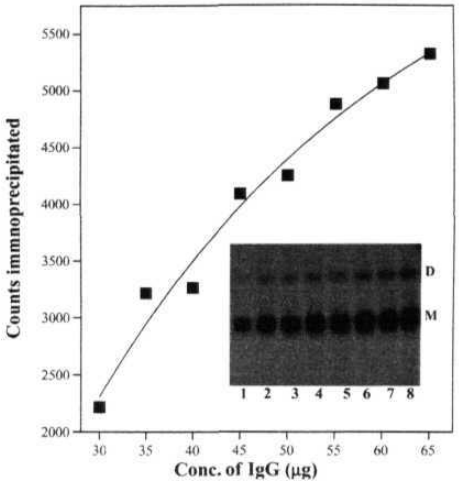


Fig. 4. Immunoprecipitation of the 237 peptide specific IgG using iodinated goat MPR 46 protein. Inset (lanes 1–8 correspond to the concentrations of IgG taken, details given under Materials and methods) shows the SDS-PAGE analysis of the precipitated receptor. M and D denote the monomer and dimer forms of the receptor specifically immunoprecipitated using the 237 IgG.

that both the MPR 300 and 46 proteins, among the different vertebrate species show biochemical and immunological characteristics similar to the well studied mammalian receptors, suggesting that both the proteins are evolutionarily conserved [2]. Further the antisera to goat receptors (MPR 300 and 46) and the antisera to MSC1 antibody have been recently used in an ELISA to quantify the receptors from different animal species [5]. In view of these available information, it is possible that the immuno-affinity matrices developed in this study can be useful in large scale isolation of the receptors from non-mammalian vertebrate animal species or from cell lines in a single step. Availability of large quantities of purified receptors will be useful to analyze their structure and functions.

Acknowledgements

The authors would like to thank the Department of Science and Technology, Government of India for the financial support in the form of a project SP/SO/D21/97. The authors are grateful to Prof. Dr. K. von Figura, Director. Biochemie II, Georg-August Universitaet, Goettingen, Germany for providing the purified synthetic peptides used in this study. The authors would also like to thank Prof. M. Ramanadham, Department of Biochemistry, University of Hyderabad, Hyderabad, for helpful discussions.

References

[1] Dahms NM, Hancock MK. Biochim Biophys Acta 2002;1572:317 40.
[2] Siva Kumar N, Udaya Lakshmi Y, Hille-Rehfeld A. von Figura K. Comp Biocheni Physiol 1999; I23B:26I 5.
[3] Udaya Lakshmi Y, Siva Kumar N, Schu P, von Figure K, Hille-Rehfeld A. Comp Biochem Physiol 2000; 127B:433 41.
[4] Siva Kumar N, von Figure K. Biosci Rep 2002;22:513–21.
[5] Suresh K, Ramanadham M, Siva Kumar N. J Biochem Biophys Methods 2002;52:111 9.
[6] Nadimpalli SK. Schmidt B, von Figure K, Hille A. FFBS Lett 1991;280:61 4.
[7] Maomi L, Jack JD. George WJ. Methods Enzymol 1989; 179:304 10.
[8] Udaya Lakshmi Y, Siva Kumar N. Biochem Mol Biol Int 1996;40:815 21.
[9] Laemmli UK. Nature 1970;227:680 5.
[10] Ansorge W. J Biocheni Biophys Methods 1985;11:13 20.
[11] Waheed A, Hille A, Junghans U, von Figura K. Biochemistry 1990;29:2449 55.