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

1. Lysosomes and lysosomal enzymes

1.1 Structure and function of lysosomes

Lysosomes are intracytoplasmic organelles defined by an acidic milieu (pH around 4.5) and surrounded by a single membrane that is present in all cell types in mammals except red blood cells. They are connected with the endocytic network and are implicated in the digestion of macromolecules. For this purpose, lysosomes contain numerous enzymes, probably more than fifty, allowing the degradation of proteins, nucleic acids, polysaccharides, lipids and their conjugates. All these substrates originate both from endocytosis and autophagy (Fig.1.1). Autophagy and endocytosis are connected lysosomal pathways because auto phagosomes receive inputs from endocytic vesicles during their maturation (Dunn, 1994; Cuervo, 2004; Majeski et al., 2004). Lysosomal acidic hydrolases comprise a variety of proteases, nucleases, glycosidases, sulfatases and lipases. Lysosomal (degradative) function also involves some other proteins such as integral membrane proteins, saposins,
and yet uncharacterized proteins. Additionally, lysosomes have been now considered as organelles that can play an important role in numerous biological processes in eukaryotes such as antigen presentation by MHC II molecules, bone resorption, tumour progression and programmed cell death (Dell'Angelica et al., 2000). In some cell types of the immune system and melanocytes, specialized lysosomes secrete cytotoxic molecules or melanin (Blott et al., 2002; Stinchcombe et al., 2004).

**Fig. 1.1 Different pathways leading to degradation by lysosomes.** Endocytosis provides lysosomes with extracellular material for digestion. It comprises three distinct processes: (i) phagocytosis, that results in the digestion of particulate material such as bacteria and occurs only in certain specialized cells like neutrophils and macrophages, (ii) pinocytosis, that allows for internalization of soluble material, and (iii) receptor-mediated endocytosis, in which the recognition of a molecule by its cognate membrane receptor is required to lead to its engulfment. The autophagic process is implicated in the degradation of cytoplasmic constituents and is generally activated in response to stress conditions. Three types of autophagy can be distinguished: (i) macro autophagy, which involves the formation of a double membrane vesicle that fuses with the lysosomal compartment, (ii) micro autophagy, which consists in the sequestration of cytosolic components directly at the surface of the degradative organelle, and (iii) chaperone-mediated autophagy that targets to the lysosomal membrane substrate proteins having a peptide motif related to KFERQ which is recognized by a cytosolic molecular chaperone. Binding to the lysosome-associated membrane protein type 2a (LAMP-2a) is then followed by translocation of the substrate protein to the lysosomal lumen.
Interest in lysosomes and lysosomal enzymes was stimulated by the existence of some 30 inherited lysosomal storage disorders in man. The enzyme defects involved in most of these disorders were identified in the 1970s (Neufeld et al., 1975). Presently, these mutations are being characterized at the level of DNA and RNA. Targeting of lysosomal enzymes is part of the more general question: how do eukaryotic cells transport proteins synthesized in the rough endoplasmic reticulum to diverse destinations? Hickman and Neufeld discovered, in 1972, that the multiple deficiency of lysosomal enzymes in I-cell disease results from a deficiency in a recognition marker that is common to lysosomal enzymes and required for targeting the enzymes to lysosomes. This observation provided the basis for many subsequent studies that eventually led to the identification of the recognition marker (a phospho-transferase enzyme that is responsible for addition of phosphate residues to mannose on the enzymes) and its receptor. The first receptor to be identified was a 215-kd protein, which recognizes mannose 6-phosphate residues in lysosomal enzymes, has been identified as an essential component of a system which in many cells allows the specific transport of lysosomal enzymes to lysosomes. It was originally identified as a cell surface receptor binding exogenous lysosomal enzymes and mediating their transfer to lysosomes along the pathway of receptor-mediated endocytosis. We now know that this receptor functions also in transport of endogenous lysosomal enzymes and that its presence in organelles that constitute elements of the secretory pathway is important for that function. The combined application of biochemical and cytological methods has significantly contributed to the present knowledge of lysosomal enzyme transport. Further, the current application of recombinant DNA methods to the study of lysosomal enzymes and their receptors is expected to provide answers to many unresolved questions.
1.2 Role of the Man 6-P/IGF-II receptor in the transport of lysosomal enzymes

The mannose 6-phosphate receptors (215 kDa protein, and the second receptor identified later, 46 kDa protein to be discussed below) are responsible for directing the transport of proteins possessing a mannose 6-phosphate recognition marker to the lysosomes. Newly synthesized lysosomal enzymes bind to the mannose 6-phosphate receptors in the trans-Golgi Network. These complexes are then localized into coated pits of the Golgi apparatus and coated vesicles are formed. These vesicles then uncoat and enter a compartment, called the pre-lysosomal compartment (PLC) or compartment of uncoupling of receptor and ligand (CURL), where the vesicle acidifies and lysosomal enzymes are released. A further sorting occurs in this compartment such that the lysosomal enzymes are directed to the lysosomes and the receptors return to the Golgi apparatus (von Figura and Hasilik, 1986; Dahms et al., 1989). Although the transport of lysosomal enzymes is understood in mammals, the precise role of the two mannose 6-phosphate receptors in this process has not been clearly defined as yet in non-mammalian species. However, only the Man 6-P/IGF-II receptor is capable of binding to mannose 6-phosphate containing proteins at cell surface to direct them to lysosomes (Sly et al., 1982; Kyle et al., 1988). Further experiments identified the recognition marker to be sensitive to oxidation (Hickman et al., 1974), mannosidase treatment (Hieber et al., 1976), deglycosylation and alkaline phosphatase treatment (Ulrich et al., 1978). Uptake of lysosomal enzymes was inhibited by a number of agents, but more strongly by fructose 1-phosphate, mannose 6-phosphate, and phosphomannans (Sando et al., 1977; Kaplan et al., 1977a; Kaplan et al., 1977b; Ulrich et al., 1978). The recognition marker was eventually identified as mannose 6-phosphate as this sugar inhibited the uptake of some enzymes by cells. These include β-galactosidase
(Distler et al., 1979) or N-Acetyl-glucosaminidase (von Figura et al., 1979) or β-glucuronidase (Natowicz et al., 1979) that are known to contain mannose 6-phosphate.

The synthesis of the mannose 6-phosphate recognition marker on lysosomal enzymes is a two step process (Lazzarno et al., 1988). Briefly, N-acetylglucosaminyl I-phosphate is transferred from UDP-N-acetyl glucosamine to the C6 hydroxyl of a mannose residue by UDP-N-acetyl-glucosamine: lysosomal enzyme N-acetyl glucosaminylphosphotransferase. The N-acetyl-glucosamine is then removed by N-acetyl glucosamine 1-phosphoexoester α-N-acetylglucosaminidase to create the mannose 6-phosphate recognition marker (von Figura and Hasilik, 1986). The transferase prefers to phosphorylate at 6th position in mannose and shows selectivity for lysosomal enzymes in their native conformation (Reitman et al., 1981).

While no consensus sequence for the transferase has been identified in lysosomal enzymes, the available evidence suggests that several small areas which are separated in the primary structure but near each other in the mature, folded protein may be the recognition signal for this enzyme (Faust et al., 1989). It is this process that is defective in I-cell fibroblasts due to a defective transferase (Hasilik and Neufeld, 1980).
1.3. Primary Structure of MPRs

Both mannose 6-phosphate receptors have been cloned and sequenced at the cDNA level from various species (Table 1.1). Both receptors are integral membrane proteins with three distinct domains, the extracytoplasmic domain, the transmembrane domain and the cytoplasmic domain (Fig. 1.3; Hille-Rehfeld, 1995; Pohlmann, 1996).
Figure 1.3: Schematic Representation of the Primary Structure of Mannose 6-phosphate Receptors. The repetitive units in the extracytoplasmic domain of MPR 300 are numbered from 1-15. The in vivo functional existence of monomeric and dimeric forms of MPR 300 and MPR 46 respectively are represented (according to Dahms et al., 1989).

1.4 M6P/IGFII receptor

The M6P/IGF-II receptor is a type I transmembrane glycoprotein consisting of four structural domains: a 40–44 residue amino-terminal signal sequence, an extracytoplasmic domain of 2264–2269 residues, a single 23 residue transmembrane region, and a carboxy-terminal cytoplasmic tail of 163–164 residues. The extracytoplasmic domain consists of 15 repeating segments of approximately 147 amino acids each, sharing 14–38% sequence identities (Fig. 1.3) (Dahms and Hancock, 2002). The 13th repeat contains an insertion of a 43 amino acid region
with homology to the fibronectin collagen binding domain that may influence ligand binding. The extracytoplasmic domain contains 19 potential N-glycosylation sites, of which at least two are utilized in forming the mature receptor of 275 – 300 kDa (Dahms and Hancock, 2002). Cysteine residues located in the extracellular repeating segments of the receptor form intermolecular disulfide bonds required for proper receptor folding. Other posttranslational modifications, such as phosphorylation and palmitoylation have also been reported for the receptor (Hille-Rehfeld, 1995; Pohlmann, 1996). The cytoplasmic domain of the receptor contains four regions that are known to be potential substrates for various protein kinases including protein kinase C (PKC), cAMP-dependent protein kinase, and casein kinase I and II (Korner et al., 1995; Dahms and Hancock, 2002). The available data indicate that receptor dimerization can occur both in vitro and in vivo. Furthermore, the observation that binding of β-glucuronidase increases the internalization rate of iodinated IGF-II and iodinated β-glucuronidase, suggests a mechanism in which receptor dimerization, resulting from the binding of a multivalent ligand, alters the kinetics of M6P/IGF-II receptor internalization at the cell surface (Byrd et al., 2000; Hassan et al., 2003; Dahms and Hancock, 2002). A truncated form of the receptor lacking primarily the intracellular and transmembrane domains has been identified in bovine serum and in the serum, urine and amniotic fluid of rats and humans (Dahms and Hancock, 2002) formation of the soluble M6P/IGF-II receptor, which retains its ligand-binding properties, is suggested to be a mechanism for receptor turnover (Clairmont and Czech, 1989, Canfield and Kornfeld, 1989). However, several lines of experimental evidence suggest that the soluble receptor functions as a carrier protein to sequester excess free IGF-II molecules in the circulation (Zaina et al., 1998; Dahms and Hancock, 2002)
1.5 Genomic organization and expression

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

1.6 MPR 46

Hoflack and Kornfeld in 1985 first discovered a cation dependent mannose 6-phosphate receptor with an apparent molecular mass of 46 kDa. The cDNA for the MPR 46 has been cloned and sequenced from several species such as human, bovine, mouse and goat (Suresh et al., 2004 and the references therein), Fugu and
xiphoporous fish (Raju thesis, 2004) Zebra fish (Suresh et al., 2006) and partially sequenced from chicken (Matzner et al., 1996). Only recently in our laboratory the full length chicken MPR 46 gene was cloned (Praveen Kumar and Siva Kumar, unpublished information). The mRNA of the human MPR 46 codes for a protein of 277 amino acids consisting of four structural domains viz., a N-terminal signal sequence of 20 or 26 amino acids, extracytoplasmic domain of 164-170 amino acids exposed at the plasma membrane or oriented to vesicle lumen, a single transmembrane domain of 20 amino acids followed by 67 amino acids of cytoplasmic domain. The short extracytoplasmic domain was shown to exhibit 14-37 % homology to individual repeats of MPR 300 (Dahms et al., 1987, Lobel et al., 1988). In contrast to this homology, there are no sequence similarities among the signal sequences, transmembrane regions and the cytoplasmic domains of the two receptors. MPR 46 contains 5 potential N-glycosylation sites, four of which are used (Wendland et al., 1991). The protein shows an absolute requirement of divalent metal ions for ligand binding and has also been classed as the cation dependent receptor (CD receptor). The carbohydrate portion contributes to 40% of the apparent molecular mass of the MPR 46. The position of cysteine residues which are most likely involved in disulfide bond formation is well conserved within the extracytoplasmic domain of MPR 46 (Lobel et al., 1988). MPR 46 is a highly conserved protein with 93-95% overall homology from mouse to man and with completely identical amino acid sequence within the cytoplasmic domain of these species. The cytoplasmic domain contains a single casein kinase-II phosphorylation site. The gene for the human MPR 46 has been localized to chromosome 12 (Pohlmann et al., 1987). The gene spans about 12 kb and consists of 7 exons (Klier et al., 1991).
<table>
<thead>
<tr>
<th>Primary Structure</th>
<th>MPR 46</th>
<th>MPR 300</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deduced amino acid sequence</strong></td>
<td>(Human MPR 46) signal sequence extracytoplasmic domain transmembrane domain cytoplasmic domain Internal repeats within extracytoplasmic domain</td>
<td>(Human MPR 46) signal sequence extracytoplasmic domain transmembrane domain cytoplasmic domain Internal repeats within extracytoplasmic domain</td>
</tr>
<tr>
<td><strong>M_r of polypeptide</strong></td>
<td>277 amino acids in total 20-26 164-170 20 67 1</td>
<td>2491 amino acids in total 40 2264 23 164 15 (~ 147 amino acids each) Fibronectin binding site in collagen showing homology in repeat 13 M6P-binding site in repeat 3 and 9 IGF-II binding site in repeat 11</td>
</tr>
<tr>
<td><strong>Post-translational Modifications</strong></td>
<td>N-glycosylation sites 30 kDa 43-46 kDa</td>
<td>270 kDa 275-300 kDa</td>
</tr>
<tr>
<td><strong>Disulfide bonds</strong></td>
<td>5 2 high mannose 2 complex</td>
<td>19</td>
</tr>
<tr>
<td><strong>Oligomerization</strong></td>
<td>3 pairs dimers, tetramers</td>
<td>3-4 pairs per repeat monomer, oligomer ?</td>
</tr>
<tr>
<td><strong>Phosphorylation</strong></td>
<td>ser 56 (CK II)</td>
<td>ser 82 (CK II)</td>
</tr>
<tr>
<td><strong>Palmitoylation</strong></td>
<td>not known</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Genomic Structure</strong></td>
<td>12 (human) 6 (murine) 12 kb 7 exons in total</td>
<td>6 (human) 17 (murine) 130 kb 48 exons in total</td>
</tr>
</tbody>
</table>
1.7 Functions of the M6P/IGF-II receptor

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

M6P-dependent transport of soluble lysosomal enzymes is a crucial step in the biogenesis of lysosomes. Newly synthesized lysosomal enzymes are carried to the lysosomes by vesicular transport from the endoplasmic reticulum, through the Golgi complex and endosomes. Initial transport steps are shared with proteins of the secretory pathway and apparently do not require specific signals. At the trans face of the Golgi complex, soluble lysosomal enzymes bind M6P receptors by their M6P-recognition signal and are subsequently transported via clathrin-coated vesicles to late endosomes (also termed prelysosomes) wherein enzyme release is triggered by the acidic interior (Hille-Rehfeld, 1995; Kornfeld, 1992). The enzymes are then transported to the lysosomes by capillary movement and M6P receptors are either targeted to the cell surface or carried back to the Golgi complex (Kiess et al., 1994; Dahms and Hancock, 2002). The segregation and transport of lysosomal enzymes is believed to be mediated by both M6P receptors as they target overlapping but distinct populations of lysosomal proteins (Kornfeld, 1992; Hille-Rehfeld, 1995; Pohlmann et al., 1995). However, several lines of evidence suggest that the M6P/IGF-II receptor is more efficient than the MPR 46 protein (also termed as the cation dependent mannose 6-phosphate receptor) in effectuating the intracellular sorting of newly synthesized lysosomal enzymes (Kornfeld, 1992; Ludwig et al., 1994., Hille-Rehfeld, 1995), It is of interest to note that, in keeping with their role in the intracellular sorting of lysosomal enzymes, the majority of M6P receptors are localized predominantly in trans-Golgi Network (TGN) and endosomal compartments, whereas only a subset of the receptors are present at the cell surface (Klumperman et al., 1993; Dahms and Hancock, 2002;). Although the exact
mechanisms of enzyme transport have yet to be determined, site directed mutagenesis experiments have shown that binding of clathrin associated proteins to an acidic-cluster-dileucine amino acid (DxxLL) motif within the cytosolic tails of M6P receptors (Fig. 1.4) is required for efficient clathrin-mediated transport of lysosomal enzymes to endosomal compartments (Boker et al., 1997; Ghosh et al., 2003). Previously, interactions between clathrin adaptor protein 1 (AP1) and the dileucine-based sorting signals of M6P receptors, in conjunction with ADP-ribosylation factor, were thought to mediate clathrin-coat assembly on vesicles budding from the TGN (Dell’Angelica et al., 2001, Dahms and Hancock, 2002). Although a role for AP1 in the transport of M6P receptors from TGN-to-endosome has not been ruled out, several recent studies have provided strong evidence that, rather than AP1, it is members of the clathrin-associated Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding (GGA) protein family, which mediates M6P receptor sorting into vesicles budding from the TGN (Fig. 1.4) (Boman et al., 2000; Dell’Angelica et al., 2000). The GGAs, which comprise three members in mammals (GGA1, GGA2 and GGA3) and two members in yeast (Gga1p and Gga2p), are monomeric modular proteins consisting of four domains: an amino-terminal VHS (for VPS27, Hrs, STAM homology) domain, a GAT (for GGA and TOM homology) domain, a connecting hinge segment, and a carboxy-terminal GAE (for γ-adaptin ear homology - a subunit of AP-1) domain (Dell’Angelica et al., 2000; Ghosh et al., 2003). The GAT domain binds ADP-ribosylation factor-guanosine 5V-triphosphate complexes and mediates recruitment of GGAs from the cytosol onto the TGN. The VHS domain interacts specifically with the DxxLL motif in the cytoplasmic tails of the M6P receptors. Mutations in the DxxLL motif impair sorting and decrease M6P receptor binding to the GGAs, indicating that this interaction is critical for sorting at
the TGN. The GAE domain binds a subset of the accessory factors that interact with the ear domain of AP-1, whereas the recruitment of clathrin triskelions to budding vesicles is most likely mediated through clathrin binding motifs of the hinge and GAE domain. Taken together, these findings suggest that GGAs are sorting proteins that recruit M6P receptors into clathrin coated vesicles at the TGN for their transport to endosomes (Dell’Angelica et al., 2000; Ghosh et al., 2003). By contrast, receptor recycling from endosomes back to the TGN does not seem to be clathrin-mediated (Draper et al., 1990; Iversen et al., 2001). Efficient retrieval of M6P receptors to the TGN appears to involve an interaction between the cytoplasmic tail of the receptor and tail binding proteins. Two such candidate tail binding proteins, phosphofurin acidic cluster sorting protein 1 (PACS-1) and MPR tail interacting protein of 47 kDa (TIP47), have been implicated in receptor recycling. PCAS-1, which binds the carboxy terminal acidic cluster of the M6P/IGF-II receptor, also interacts with AP-1 (Diaz et al., 1998; Wan et al., 1998; Ghosh et al., 2003). Anti-sense mediated depletion of PACS-1 or over expression of a mutant PACS-1 that binds cargo tails but not AP1, results in a shift in M6P/IGF-II receptor distribution away from the perinuclear region towards peripheral endosomal structures, as observed in cells lacking AP-1 (Crump et al., 2001; McKinnon et al., 2001). These findings suggest that PACS-1 may act as a connector between the M6P receptors and AP-1 to facilitate recycling of the receptors from early endosomes to the TGN. A role for TIP47 in M6P receptor retrieval is supported by evidence that antibody-mediated reduction of endogenous TIP47 can inhibit M6P receptor transport from late endosomes to the TGN. Optimal TIP47 function depends on binding to Rab9, a late endosome GTPase that increases the affinity of TIP47 for M6P receptors (Diaz et al., 1998; Carrol et al., 2001; Dell’Angelica et al., 2001). These data indicate that PACS-
1/AP-1 likely mediates receptor recycling from early endosomes, whereas TIP47/Rab9 recycles receptors from late endosomes (Dahms and Hancock, 2002; Ghosh et al., 2003). However, the relative contribution of these two pathways to the total M6P receptor retrieval/trafficking remains to be determined.

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

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

Cell surface M6P/IGF-II receptors, but not CD-M6P receptors, mediate endocytosis of a variety of M6P-containing ligands for their subsequent clearance or activation. The M6P/IGF-II receptor plays a general role in the recapture of endogenous, newly synthesized lysosomal enzymes which escape sorting at the TGN or that have been actively exported by the CD-M6P receptor (Koster et al., 1994; Hille-Rehfeld, 1995). Endocytosis of lysosomal enzymes by the M6P/IGF-II receptor serves as a mechanism to facilitate degradation of extracellular matrix proteo-glycans or to transfer enzymes from one cell type to another (Roff et al., 1983; Kornfeld, 1992). There is evidence that this receptor also mediates the internalization and subsequent degradation or activation of proliferin (a prolactin-related murine protein) (Lee et al.,
glycosylated human LIF (Blanchard et al., 1999), renin precursor (Saris et al., 2001) and epidermal growth factor receptor (Todderud et al., 1988). The internalization process appears to involve the formation of clathrin coated vesicles in a process mediated by the interaction between clathrin associated adaptor protein AP2 and the single tyrosine-based internalization motif YSKV, located on the cytoplasmic tail of the M6P/IGF-II receptor (Kornfeld, 1992; Dahms and Hancock, 2002).

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

In addition to lysosomal enzymes, several other proteins have been demonstrated to possess mannose 6-phosphate and to be capable of binding to the M6P/IGF-II receptor. In general, the lysosomal enzymes possess complex oligosaccharide structures that contain phosphorylated mannose residues, and thereby exhibit distinct binding to the receptors. On the other hand some prohormones possess only one or two mannose 6-phosphates and bind with lower affinity (Fischer et al., 1982; Dong et al., 1990). This may explain the phenomenon whereby the vast majority of lysosomal enzymes are sorted directly from the Golgi apparatus to the lysosome, while the prohormones often escape the cell and are later recaptured. Some examples are pro-transforming growth factor TGF-β, proliferin, growth factors, and thyroglobulin (the precursor to thyroid hormones). The cell surface M6P/IGF-II receptor is also believed to facilitate activation of the TGF-β precursor (Dennis et al., 1991; Ghahary et al., 1999), the proform of a hormone which regulates differentiation and growth of many cell types. The latent pro-TGF-β, one component of which contains M6P residues, is secreted from cells and stored in the extracellular matrix as an inactive precursor complex that can be converted to its active form only after
processing (Munger et al., 1997; Villevalois-Cam et al., 2003). Although TGF-β activation has been reported to be mediated by the matrix glycoprotein thrombospondin-1 (Crawford et al., 1998), several lines of evidence suggest a role for plasmin-mediated activation of TGF-β following its binding to cell surface IGF-II/M6P receptors (Dennis et al., 1991; Ghahary et al., 1999). Moreover, recent data, which demonstrated the ability of plasminogen and the urokinase-type plasminogen activator receptor (uPAR) to bind the M6P/IGF-II receptor at regions distinct from the M6P binding pockets, support a plausible model in which binding of urokinase plasminogen activator to a uPAR that is complexed to the receptor, facilitates conversion of plasminogen to plasmin, which in turn proteolytically activates receptor bound TGF-β precursor (Godar et al., 1999; Ghosh et al., 2003).

Pro-transforming Growth Factor- β

The presence of two phosphorylated oligosaccharides at Asn 82 and 136 on pro-TGFβ1 has recently been determined (Purchio et al., 1988) and it has been demonstrated that this protein binds to the M6/I GF-II receptor in a mannose 6-phosphate inhibitable manner (Kovacina et al., 1989). The presence of a latent, precursor form of TGF-β has previously been suggested (Nilsen-Hamilton et al., 1980; Wakefield et al., 1988 ;). It is of interest that while there are multiple forms of TGFβ (Derynck et al., 1985; Obberghen-Schilling et al., 1987; Marquardt et al., 1987; Dijke et al., 1988) only the first form, TGFβ, has been shown to possess the lysosomal enzyme recognition marker mannose 6-phosphate). The precise role of the TGFs is to play a role in the production of collagen matrix and its interaction with cells (Ignotz et al., 1987; Montesano et al., 1988) and in the control of proliferation, differentiation, and transformation (Sporn et al., 1986; Keski-Oja et al., 1987). The
physiological role of the binding of pro-TGF-1 to the M6P/IGF-II receptor is also not known at this time, but it may play a role in the processing of this prohormone.

**Proliferin**

The next growth factor that possesses a mannose 6-phosphate moiety and can bind to the mannose 6-phosphate receptor is proliferin (Lee et al., 1988). Proliferin is an approximately 25 kDa glycoprotein growth factor which is secreted by mouse placental tissue and a number of growing mouse cell lines (Lee et al., 1988). It is related to the placental lactogens (Lee et al., 1988) and shows a 31% identity with prolactin. At this time the only receptor identified for this molecule is the M6P/IGF-II receptor, which it binds with a high affinity (kd > 1-2 nM) which is dependent upon glycosylation and phosphorylation and can be inhibited by mannose 6-phosphate (ki ~ 10µM) or anti-M6P/IGF-II receptor antibodies (Lee et al., 1988). The functional significance of the proliferin-receptor interaction is still not fully understood.

**Thyroglobulin**

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

1.8a. M6P and human IGF-II interaction

The non-glycosylated 67 amino acid IGF-II peptide is the best-characterized non M6P-containing ligand of the M6P/IGFII receptor (O'Dell et al., 1998; Dahms and Hancock, 2002). Several lines of experimental evidence over the last decade have clearly indicated that IGF-II plays a crucial role in mammalian growth by influencing fetal cell division and differentiation (Ludwig et al., 1995; O'Dell et al., 1998). Interestingly, the growth promoting effects of IGF-II are believed to be mediated by its ability to bind IGF-I and/or insulin receptors and the role of the M6P/IGF-II receptor is to divert excess IGF-II for its degradation in lysosomes. This is supported in part, by experimental data which have shown that i) antibodies against the receptor do not inhibit the mitogenic effect of IGF-II (Kiess et al., 1987), while IGF-I receptor blocking antibodies impair IGF-II action in various cell culture systems (Furlanetto et al., 1987); (ii) viable IGF-II-deficient mice are 40% smaller than their wild type siblings (Baker et al., 1993); and (iii) IGF-II mutants with a weak affinity for the M6P/IGF-II receptor but a high-affinity for the IGF-I receptor induce biological responses (i.e., stimulation of DNA synthesis in BALB/c 3T3 cells and glycogen synthesis in Hep G2 cells) in correlation with their affinity for the IGF-I receptor (Sakano et al., 1991). On the other hand, IGF-II recognition and internalization by the M6P/IGF-II receptor is postulated to be a general mechanism used to modulate circulating levels of IGF-II by targeting it for lysosomal degradation. This is substantiated, at least in part, by gene targeting studies which have shown that mice lacking the M6P/IGF-II receptor exhibit fetal overgrowth, elevated levels of circulating IGF-II and perinatal lethality as a consequence of major cardiac abnormalities (Lau et al., 1994; Wang et al., 1994). Interestingly, this phenotype can be completely
rescued by simultaneous deletion of (knocking out) either the IGF-II peptide or IGF-I receptor gene (Ludwig et al., 1996), thus suggesting that the lethality observed in M6P/IGF-II receptor-deficient mice is caused by an over stimulation of the IGF-I receptor by excess IGF-II. While the function of the M6P/IGF-II receptor in IGF-II clearance is well accepted, its role in mediating any biological actions of the growth factor remains controversial. Several studies, however, indicate that binding of IGF-II to the M6P/IGF-II receptor can induce specific responses, including increased amino acid uptake in muscle cells (Shimizu et al., 1986), glycogen synthesis in hepatoma cells (Hari et al., 1987), exocytosis of insulin from pancreatic cells (Zhang et al., 1997), cell proliferation in K562 erythroleukemia cells (Tally et al., 1987), increased gene expression in spermatocytes (Tsuruta et al., 2000), motility of human rhabdomyosarcoma cells (Minniti et al., 1992), migration of human extravillous trophoblasts (McKinnon et al., 2001), stimulation of Na+/H+ exchange and inositol triphosphate production in canine kidney cells (Rogers et al., 1988) and calcium influx (but not cell proliferation) in primed BALB/c3T3 fibroblast cells (Kojima et al., 1988; Matsunaga et al., 1988). Receptor specificity in most cases was confirmed by the use of a rather selective M6P/IGF-II receptor analogue, receptor antibodies which mimic/block IGF-II effects, or evaluating the effects in a system which lacks IGF-I receptors (McKinnon et al., 2001; Minniti et al., 1992). Given that the cytoplasmic tail of the M6P/IGF-II receptor lacks a kinase domain, the intracellular mechanisms by which the receptor can mediate such biological effects remain unclear. However, a number of studies in cell-free experimental systems and a few studies in living cells have provided evidence for an interaction of the M6P/IGF-II receptor with heteromeric G proteins (Ikezu et al, 1995; Minniti et al, 1992). By comparing the sequence of the human M6P/IGF-II receptor with that of mastoparan,
a small peptide in wasp venom that can directly activate Gi and Go proteins (Higashijima et al., 1990), it has been shown that a 14 residue amino acid residue (Arg2410–Lys2423) in the cytoplasmic region of the M6P/IGF-II receptor can mediate Gi₀ activation. This is supported by evidence that adenylate cyclase activity was inhibited by IGF-II in COS cells transfected with constitutively activated Gi₀ (Nishimoto et al., 1990; Okamoto et al., 1990; 1991) and wild-type M6P/IGF-II receptor cDNAs, but not with M6P/IGF-II receptors lacking Arg2410–Lys2423. Furthermore, homology was noted between the C-terminal Ser2424–Ile2451 region of the M6P/IGF-II receptor and part of the pleckstrin homology domain of several proteins that bind G₉ and inhibit its stimulatory action on adenylate cyclase activity (Ikezu et al., 1995). At the functional level, there is evidence to suggest that IGF-II, acting via a Gi protein, can stimulate Ca^{+2} influx in 3T3 and CHO cells (Kojima et al., 1988; Matsunaga et al., 1988), increased exocytosis of insulin from the pancreatic cells (Zhang et al., 1997) and promote migration of extravillous trophoblast cells (McKinnon et al., 2001). Additional findings have shown that M6P/IGF-II receptor-activated G protein can lead to PKC-induced phosphorylation of intracellular proteins (Zhang et al., 1997), stimulation of MAP kinase pathway and/or decrease in adenylate cyclase activity (McKinnon et al., 2001). These results, taken together, suggest that the M6P/IGF-II receptor may mediate certain biological effects of IGF-II, most likely via activation of a G-protein coupled pathway. However, given the evidence that M6P/IGF-II receptor, under certain conditions, failed to interact with G protein or to couple Gi₀ (Korner et al., 1995), the overall significance of the receptor - G protein interactions under in vitro conditions and its relevance to normal physiology are a matter of speculation.
1.8b. M6P/IGF-II receptor and cancer

The ability of the receptor to modulate levels of the mitogen IGF-II (O'Dell et al., 1998), to facilitate activation of the growth inhibitor TGF-β (Dennis et al., 1991; Ghahary et al., 1999) and to regulate targeting of lysosomal enzymes to lysosomes (Hille-Rehfeld, 1995; Kornfeld, 1992), suggests that the receptor could act as a tumour suppressor. This is supported by data which showed that over expression of the receptor acts as a growth inhibitor under both in vitro and in vivo conditions (O’Gorman et al., 2002), whereas loss of receptor function is associated with progression of tumorigenesis (Oates et al., 1998; DaCosta et al., 2000). Frequent loss of heterozygosity at the M6P/IGF-II receptor locus has been reported for a variety of cancers including liver (De Souza et al., 1995; Yamada, et al., 1997), breast, ovarian (Hankins et al., 1996; Chappell et al., 1997; Rey et al., 2000) and lung (Kong et al., 2000). In some of these cases, somatic mutations in the remaining parts of the receptor allele have been identified, the majority of which disrupt M6P and/or IGF-II binding properties (Byrd et al., 1999; Devi et al., 1999). While only a single point mutation (P2379T) in the cytosolic tail has been identified, nine human cancer-associated mis-sense mutations of the extracytoplasmic region of the MPR/IGFII have been reported: two are located in M6P recognition domain 9 (C1262S, G1296R), three are in domain 10 (Q1445H, G1449V, G1464E), and four are in IGF-II binding domain 11 (G1564R, I1572T, A1618T, and G1619R). Significantly, with the exception of G1464E, all of the missense mutations (C1262S, Q1445H, G1449V, G1464E, and I1572T) that have been characterized to date result in receptors with altered M6P and/or IGF-II binding properties, supporting the hypothesis that loss of normal M6P/IGF-II function contributes to carcinogenesis. However, to gain new insights further functional studies need to be carried out.
The M6P/IGF-II receptor has also been reported to bind retinoic acid to induce changes in cell shape, growth inhibition and apoptosis (Kang et al., 1997; 1999). The ability of the receptor to recognize many functionally distinct ligands illustrates not only the multifunctional role of the receptor, but also raises the possibility of its involvement in a myriad of important physiological functions.

1.9. MPRs in the invertebrates and an evolutionary perspective.

1.9a. MPRs in the invertebrates

Although the MPR proteins clearly have a major role in lysosomal enzyme trafficking in the vertebrate cells, their role in invertebrate systems is not well defined. Lysosomal enzymes are successfully targeted in lower eukaryotes such as *Saccharomyces*, *Trypanosoma*, and *Dictyostelium*, without the aid of identifiable MPRs. Both types of MPRs are present in the invertebrates such as the molluscs, (Siva Kumar and von Figura, 2002) but they are not well characterized, and thus, we do not yet know how the MPR genes have evolved. Recently, a *Drosophila* protein (lysosomal enzyme receptor protein, LERP) that is structurally and functionally related to the mammalian CI-MPR was identified (Dennes et al., 2005). LERP mediates lysosomal enzyme targeting and rescues the missorting of lysosomal enzymes that occurs in MPR-deficient mammalian cells. Interestingly, however, the residues that are involved in M6P recognition in mammalian MPRs are not conserved in the LERP and it does not bind to the multimeric M6P ligand phosphomannan. The nature of the interaction between LERP and mammalian lysosomal enzymes has not yet been elucidated. Above the molluscs in the echinodermites in this study we also identified the receptors and extensively characterized the MPR 46 protein. The slime mold *Dictyostelium discoideum* produces a novel methyl-phosphomannose sequence on some of its lysosomal
enzymes that can be recognized in vitro by the mammalian CI-MPR (not the CD-MPR). However, despite the presence of a GlcNAc-Phospho transferase that recognizes α1–2-linked mannose residues, no receptor for the phosphorylated mannose residues has been found in these organisms. Notably, although this phosphotransferase does not show the specific recognition of lysosomal hydrolases as seen with the mammalian enzyme, it produces another transferase that selectively adds GlcNAc-1-phosphate to serine residues. In contrast to this situation, the protozoan *Acanthamoeba* produces a GlcNAc-phospho transferase that does show specific recognition of lysosomal enzymes (Gabel et al., 1984; Couso et al., 1986). Although some of these organisms show evidence for an “uncovering” enzyme, no definable MPR has yet been found.

**1.10 An evolutionary perspective and scope of the present study**

According to Darwin’s theory of evolution (Darwin et al., 1859), natural selection will serve to retain favourable traits and eliminate unfavourable ones. For this reason we generally assume that a conserved function or sequence of a protein in different organisms is important to that organism. With respect to the M6P/IGF-II receptor protein that has been shown to bind not only lysosomal enzymes but also IGF-II, we would expect the IGF-II binding capacity to play an important role in mammals. Indeed by experimentation, it has been demonstrated that the primary purpose of the binding of IGF-II by the M6P/IGF-II receptor is to degrade IGF-II and this activity is essential for normal mammalian development. The acquisition of an IGF-II binding site by the receptor appears to have occurred after the divergence of marsupial and placental mammals from their common ancestor with egg-laying mammals, and it has been suggested that this acquisition was a major factor in driving the evolution of an imprinted M6P/IGF-II receptor in some mammals (Killian et al., 2000). However,
a high affinity IGF-II binding site has been described in the receptor of a teleost fish, early vertebrate, (Mendez et al., 2001), raising the possibility that IGF-II binding was an ancestral property of the receptor and also it may reflect an evolutionarily ancient aspect of lysosome biogenesis that predates the M6P-dependent trafficking of lysosomal enzymes seen in present-day mammals. It also raises the question of how, and when, the involvement of the M6P-specific system in lysosome biogenesis evolved. Moreover, the homologies exhibited by the domain structures and ligand-binding activities of some of the vertebrate MPRs raise questions about their phylogenetic origin, evolutionary history, functional significance in vivo and the specific evolutionary divergence point at which the complete MPR system came into existence. Answers to these questions can only be known by carrying out detailed comparative studies of the receptors from different non-mammalian vertebrates, invertebrates using animal models as well as available specific cell types.

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

The following sentence gives some details about the multifunctional nature of the known M6P/IGF-II receptors. Among the two MPRs only the mammalian MPR 300 has so far been shown to be a multifunctional protein which, in addition to binding mannose 6-phosphate containing lysosomal enzymes, also binds insulin-like growth factor-II (IGF-II), other ligands such as retinoic acid and thyroglobulin. The receptor has been classed as the M6P/IGF-II receptor. In humans, it has been shown that the M6P/IGF-II receptor plays an important role in controlling the extracellular level of the IGF-II by mediating its binding at the cell surface and delivery to lysosomes. The luminal ligand binding domain of MPR 300 contains 15 internal repeats (cassettes), which are homologous to each other and to the single luminal domain of MPR 46. It is therefore, of interest to study the evolution and functions of these receptor proteins in order to analyze their biochemical and ligand binding properties. MPR 300 proteins purified from a number of species such as, human, rat, bovine, and opossum exhibited IGF-II binding abilities and the IGF-II binding domain in mammals has been localized to repeat 11 of this multifunctional receptor. The biochemical and immunological properties of the purified MPR 300 protein from other animal species such as goat, chicken, garden lizard, fish (non-mammals) and unio (invertebrate) resemble those of the mammalian receptor. Further a partial cDNA clone for the fish MPR 300 has revealed that the receptors in the vertebrates contain conserved cassette structures in the luminal domain and the mannose 6-phosphate binding region in the third cassette exhibits extensive sequence homology among all the vertebrates, suggesting that this protein is evolutionarily conserved (Udaya Lakshmi et al., 2000). However, in a recent study it has been shown that the MPR 300 from the early non-mammalian vertebrate, fish binds human IGF-II under specific
conditions (Mendez et al., 2001). This recent observation led us to believe that possibly the IGF-II binding property and the multifunctional nature of the MPR 300 may also be conserved in the other non-mammalian vertebrates such as chicken and reptiles.

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

In the present study the following objectives are studied in detail

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

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

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

MATERIALS

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

METHODS

Cell culture

Chicken embryonic fibroblast (CEF) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum. Cells were grown in standard flasks as well as in 3cm petri plates in an incubator at 37°C in an atmosphere containing 5% CO\textsubscript{2} as described (Matzner et al., 1996).

Extraction of membrane proteins from CEF cells

Confluent monolayers grown in 90 cm petri plates were scraped with the help of a cell scraper, and the cell pellet was collected by centrifugation in a Biofuge stratos centrifuge, using a microliter rotor at 2991 \times g for 10 min. The cells were suspended in 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge, using a
fixed angle 80Ti rotor at 161,280 × g for 35 min. The pellet obtained at this step was dissolved in 50 mM imidazole-HCl buffer pH 7.0 containing 0.5% Triton X-100, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and re-centrifuged as described above. The MPR 300 protein from this membrane extract as well as from the chicken liver membrane extract was purified to homogeneity by phosphomannan affinity chromatography (PM gel) as described (Suresh et al., 2006).

**Protein assay**

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

**In vitro iodination**

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

**Affinity chromatography on Tg-Sepharose**

Tg-Sepharose gel and BSA coupled to affigel-10 were equilibrated at 4°C with 50 mM Tris-HCl, 0.9% NaCl, 0.1% BSA, 5 mM CaCl$_2$ pH 7.4 containing 0.05% Triton X-100 (buffer A) as described (Lemansky et al., 1992) In brief, $^{125}$I, radiolabeled CEF M6P/IGFIIR 300 (10, 00,000 cpm) in buffer A (100 µl), was loaded on to the Tg affinity gel (200 µl) or onto the BSA gel (200 µl) at a flow rate of 2.5 ml/min. Unbound proteins were removed by washing with 10 volumes of wash buffer (buffer A). Bound proteins were eluted with buffer A containing 5 mM glucose 6-phosphate.
followed by 5 mM mannose 6-phosphate. Column fractions were TCA precipitated, separated by SDS-PAGE and the bands visualized by autoradiography.

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

Radio iodinated Tg was diluted in buffer A to give final concentrations ranging from 8 to 86 nM. Cells were grown in 12 well culture plates, to 80-85 % confluency and incubated with 10 different concentrations (8 to 86 nM) of $^{125}$I-Tg for 90 min at 4$^\circ$C in binding buffer (buffer A without TritonX100) in the presence of 2 mM mannose [to avoid interference with the possible binding of Tg by its mannose residues]. In a separate experiment, non-specific binding was determined in the presence of 2 mg/ml non-radioactive Tg. After the incubation, the cells were washed in PBS containing 1% BSA five times, and lysed with lysis buffer (1% Triton X- 100, 50 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, 0.02% NaN$_3$ supplemented with 1 mM PMSF, and 1 mM EDTA as protease inhibitors on ice for 30 min. The cell suspension was centrifuged at 100 $\times$ g for 2 min. The supernatant was discarded and pellets were counted in a gamma counter, and the amount of bound Tg was calculated and normalized to the concentration of the membrane protein. Analysis was performed in duplicates and the average values presented. Saturation and scatchard plot analysis were carried out using the details in (www.graphpad.com).

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

The binding and internalization of $^{125}$I-Tg in CEF cells was compared by preincubating the cells with mannose 6-phosphate (5 mM), glucose 6-phosphate (5 mM), goat MPR 300 IgG (10 µg), unlabeled Tg (200 nM), rabbit IgG (5 µg) and human IGF-II (2 µg). Binding analysis was carried out as described above. For internalization, cells were grown to 80-85 % confluency in 6 well culture plates. The cells were rinsed five times with DMEM containing 20 mM HEPES to remove the
residual serum. $[^{125}\text{I}]$ Tg (5,00,000 cpm) was then added to the cells in 1 ml of serum-free medium and incubated for 30 min at $37^\circ\text{C}$. At the end of incubation the cells were washed six times with serum-free medium, three times with medium containing 1 mg/ml bovine serum albumin and five times with phosphate-buffered saline. The volume of each wash was 1 ml. After washing the cells, they were incubated with 0.5 ml of lysis buffer as above. Radioactivity in the lysate was measured in a gamma counter.

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

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

**Ligand blotting**

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

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

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

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

In vitro fluorochromation of Tg

Bovine Tg, 5 mg was incubated with 550 µl borate buffer (50 mM, pH 9.0) and 100 µl fluorochrome solution of fluoresce-inisothiocyanate [5 mg FITC dissolved in one ml
of DMSO] overnight at 4°C. Free fluorochrome was removed by desalting using a Sephadex G-25 gel as described above.

**Immunofluorescence microscopy**

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

RESULTS

*Tg-Sepharose affinity chromatography*

The MPR 300 protein from CEF cells and chicken liver was purified to homogeneity (data not shown) and radio iodinated as described under methods. These proteins were separately applied on to Tg-Sepharose gel. (BSA–affigel-10 matrix prepared in the laboratory was used as a control gel). The gels were processed as described under methods and the column fractions and eluates were analysed by SDS-PAGE. The CEF cell M6P/IGFIIIR and the chicken liver MPR 300 were both bound on Tg-Sepharose gel and could be specifically eluted using 5 mM M6P (Fig 2.1A and B).

[Under similar conditions the goat purified and radio iodinated MPR 300 was bound to the gel, data not shown]. When the iodinated receptors were applied on BSA-affigel, all the radioactivity could be recovered in the unbound fraction suggesting no binding of the receptors to BSA (data not shown).

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

*Characterization of Tg binding to Chicken embryonic cells*

To characterize the Tg binding to the CEF cells, these were incubated with radio iodinated Tg with or without 3 µM non-radioactive Tg at 4°C. In the absence of non-radioactive Tg, the amount of cell-bound [125I] Tg increased with increasing amounts of free radiolabeled Tg exhibiting saturation kinetics (Fig 2.3A open circles). In the
presence of non-radioactive Tg, the cell-bound radioactivity was lower, and increased in a linear fashion (Fig 2.3A, triangles). The specific binding of $[^{125}\text{I}]$ Tg to the surface of CEF cells was calculated (Fig 2.3B) by subtracting non-specific binding (Fig 2.3A, triangles) from total binding (Fig 2.3A, open circles). Saturation of specific Tg binding to CEF cells was reached at 66 nM of Tg. Scatchard analysis revealed a dissociation constant of 33 nM (Fig 2.3C).

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

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

**M6P/IGFIIIR interacts with thyroglobulin in vitro**

The binding of the radiolabeled Tg to the CEF cell MPR 300 was analysed in a ligand blot experiment. From Figure 2.5A, it is apparent that a band could be visualized in the region of the MPR 300 protein suggesting binding of the iodinated Tg to the CEF cell MPR 300 protein. The binding capacity of Endo H, PNGase F and
alkaline phosphatase treated Tg to MPR 300 protein was demonstrated *invitro* using the purified M6P/IGFIIR as a probe in a far Western blot analysis. Tg untreated with enzymes, showed clear recognition by the receptor (Fig 2.5B, lane 1). Treatment of the Tg with Endo H, PNGase F or with phosphatase completely abolished the binding (Figure 2.5B, lanes 2, 3 and 4) suggesting the importance of M6P moieties for specific binding.

**Degradation of Tg in chicken embryonic fibroblast cells**

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

**Fluorescence microscopy analysis**

CEF cells were incubated with Tg to study binding as well as internalization using fluorescence microscopy. Incubation of CEF cells with Tg at 4°C followed by immunolabeling, revealed a patched staining pattern along the entire cell surface (Fig 2.6A). Incubation with FITC-labeled Tg for 30 min at 37°C, showed fluorescence of Tg in endocytic vesicles. Tg containing vesicles were distributed throughout the cytoplasm of CEF cells. For identification of those vesicles as early or late endocytic compartments, CEF cells were immunolabeled with antibodies against the lysosomal enzyme arylsulfatase A (Fig 2.6B). It is well established in mammals by morphology and by the immunocytochemical detection of lysosomal
enzyme arylsulfatase A marker protein, these Tg-containing vesicles were identified as prelysosomal or early endosomes. The results indicated that Tg was bound to the plasma membrane of CEF cells at 4°C, and was internalized when incubations were done at 37°C. To confirm that M6P/IGF-IIR and endocytosed Tg are colocalized and present in the early or late endosomal compartments, cells were immunolabeled with antibodies against M6P/IGF-IIR and examined by immunofluorescence microscopy. As shown in Fig 2.6C, colocalization is distinctly observed and M6P/IGF-IIR is located together with Tg in vesicles surrounding the nucleus corresponding to late endosomes.
**Figure: 2.1 Affinity chromatography on Tg-Sepharose gel. A)** Purified and radio iodinated CEF MPR 300. 7.5% SDS-PAGE analysis. Lane 1, unbound fraction, lane 2, wash, lane 3, glucose 6-phosphate eluate and lane 4, mannose 6-phosphate eluate (specific elution).

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

**Figure: 2.2 Time course for binding and internalization of \(^{125}\text{I-Tg}\) by CEF cells.**

CEF cells were grown in 12 well culture plates and incubated with 1, 00,000 cpm \(^{125}\text{I-Tg}\) in binding buffer at different time intervals at 4°C as well as 37°C, washings and lysis was done as described in methods. Solid line and closed circles are surface-associated, dotted line and open circles are Internalized \(^{125}\text{I-Tg}\) respectively.
Figure 2.1

Figure 2.2
Figure 2.3: Saturation binding assay of Tg binding on Chicken embryonic fibroblast cells. Cells were incubated with increasing amounts of radio iodinated Tg at 4°C.

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

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

C). Scatchard plot analysis demonstrated a dissociation constant of 33 k_d.
Figure 2.3
Figure 2.4: Binding and internalization of $[^{125}\text{I}]\text{Tg}$ by CEF cells. Cells were incubated with $[^{125}\text{I}]\text{-Tg}$ without any ligand and with different ligands shown in as described under methods. The % of radioactivity bound (panel A) and internalized (panel B) was analysed.
Figure 2.4
**Figure: 2.5 In vitro binding of Tg to CEF MPR 300.**

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

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

**C).** Mannose 6-phosphate dependent endocytosis of thyroglobulin by CEF cells. Thyroglobulin was incubated with CEF cells in the presence and absence of 5 mM mannose 6-phosphate. From these cell lysates, Tg was immunoprecipitated, analyzed by 7.5% SDS-PAGE and protein bands detected by fluorography. Lane 1, proteolysis of Tg can be seen in the absence of 5 mM mannose 6-phosphate while no such effect was seen in presence of 5 mM mannose 6-phosphate (lane 2).
**Figure 2.6: a) Binding of Tg by CEF cells.** Cells were incubated with Tg at 4°C, and were immunolabeled with monoclonal antibodies against Tg or mouse IgG (Isotopic control). Tg binding was seen along the cell surface (arrowheads). (A-D, see details in figure, *(Bar 11.7 µm)*).

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

**c) Colocalization of M6P/IGFIIR and FITC-Tg within endocytic compartments of CEF cells.** (A) Internalization of FITC labeled Tg at 37°C (B) After fixation, cells were immunolabeled with polyclonal anti-Goat MPR 300 antibody and TRITC-labeled secondary antibody. (C) Co-localization of M6P/IGFIIR (A) and FITC-Tg (B) within early or late endosomal compartments of CEF cells. (C) Merge of A and B. (See details figure A-D) *(Bars, 11.5µm).*
Figure 2.6
DISCUSSION

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

The mammalian MPR 300 protein was shown to play a role in the clearance and activation of hormones and growth factors, and is able to bind and internalize IGF-II for its delivery to lysosomes in contrast to the second P-type lectin, MPR 46 which is involved in sorting of lysosomal enzymes only. The cDNA clones and partial sequence analysis of the putative receptors have been described in the chicken (Matzner et al., 1996) and the levels of the receptor proteins in different tissues of mammals and chicken vary (Suresh et al., 2006). Further structural analysis of the fish, chicken and the mammalian receptors revealed that the M6P/IGF-II receptor
protein exhibits a conserved cassette structure in the amino terminal region throughout the vertebrates (Udaya Lakshmi et al., 2000). The focus of research in our laboratory is to understand the structure and function of the putative MPR proteins in the animal kingdom. Although it has been well established that the mammalian MPR 300 protein is a multifunctional protein and capable of binding IGF-II and other ligands, it was not clear until recently whether this property is conserved in non-mammalian vertebrates. In a recent study we have shown that purified goat and CEF cell MPR 300 protein are able to bind IGF-II suggesting the possible multifunctional nature of these receptors (Suresh et al., 2006). Since we characterized that the CEF cell MPR 300 protein binding to IGF-II, we set out to analyse whether the multifunctional nature of the MPR 300 protein is also conserved in evolution using the bovine Tg that has been well characterized (Allen et al., 1993; Eduardo et al., 1987) and with the aim to find the possible binding receptor for thyroglobulin on CEF cells. To compare our results with the literature information we used bovine Tg in our studies. In the present study, the first evidence that the purified chicken M6P/IGF-II protein (purified from CEF cells and chicken liver) binds to bovine-Tg in a M6P dependent manner was obtained by passing the radiolabeled receptors on Tg-Sepharose gel. The specificity of the binding has been further substantiated by its elution from the gel by mannose 6-phosphate but not by glucose 6-phosphate. These data suggest that the receptors from both CEF cells as well as from the chicken liver can bind on Tg-gel suggesting Tg as an additional ligand to this receptor protein in CEF cells. [Since we have been using the goat receptors as a mammalian model for our studies, we have also checked if the purified goat MPR 300 protein can bind Tg, and we found that this mammalian receptor also binds Tg, like the human receptor in a M6P dependent manner, [data not shown].
Thyroglobulin binds M6P/IGF-II protein with high affinity and reaches saturation at 66 nM with a $K_d$ value of 33 nM. Preincubation of CEF cells with 5 mM mannose 6-phosphate, anti-goat MPR 300 antibody showed about 80% inhibition whereas glucose 6-phosphate, human IGF-II did not show any remarkable inhibition on Tg binding and internalization. In a competitive binding assay with unlabeled Tg there is decline in surface bound radiolabeled Tg showing the competition between labeled and unlabeled Tg to bind M6P/IGFII (data not shown). Prior treatment of Tg with Endo H, PNGase F and alkaline phosphatase showed about 90% inhibition. In far-Western blot analysis deglycosylated, dephosphorylated Tg did not show binding with M6P/IGFII compared to the untreated Tg. These data suggest that glycosylation and phosphorylation of Tg is important for binding to M6P/IGF-II protein.

Immunofluorescence microscopy studies revealed that the Tg can bind to the M6P/IGF-II protein on the CEF cell surface as detected using a monoclonal antibody to the Tg. Furthermore, experiments with fluorescent labeled Tg in fluorescence microscopy also reveals the clear distribution and colocalization of the Tg with the M6P/IGF-II protein in the cytoplasm and perinuclear region. The detection of arylsulfatase A enzyme in CEF cells in endocytic compartments suggests that these vesicles are late endosomes or prelysosomes because lysosomal enzymes distribution was consistent with the fact that the late endosome and prelysosomal network is the compartment where newly synthesized acid hydrolases containing the M6P marker are sorted from proteins that will be secreted. It is well established in mammals that the M6P/IGF-II protein is localized in living cells to different endocytic compartments and the detection of the arylsulfatase A enzyme in these endocytic compartments along with FITC-Tg strongly supports the role of CEF M6P/IGF-II
protein in internalization of this specific ligand. Our experimental data shown in Figure 6, clearly support the idea that Tg taken up by the CEF cells via the receptor is routed to the lysosomes through the intracellular compartments of the endocytic pathway and transported from the early and late endosomes to the prelysosomal vesicles like the arylsulfatase A.

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

Our studies clearly demonstrate that the CEF cells endocytose Tg through the M6P/IGF-II receptor present on the cell surface which is then targeted to the lysosomes for degradation. However it remains to be established whether the results found with the CEF cells, can be extrapolated to the chicken liver hepatocytes. Additionally, it is also not known whether the receptor is present in the thyroidal cells and to what extent? Further studies are necessary to gain new information on these which might give the necessary inputs to study this receptor and its interaction with Tg in this model organism. In summary, this is the first report to provide evidence that the CEF MPR 300 protein can also bind Tg in addition to M6P containing hydrolases and IGF-II. The study thus provides new information on the multifunctional nature of the chicken MPR 300 protein and provides a strong functional significance for the conserved cassette structure of the MPR 300 protein in the vertebrates. Other researchers have already shown that the fish MPR 300
protein also binds IGF-II (Mendez et al., 2001). However it remains to be established whether the fish receptor like the CEF cell receptor can also bind Tg. Table 2.1 describes the comparison of binding abilities of M6P/IGF-II protein from mammalian and non-mammalian species with a number of ligands. Expression of the fugu receptor gene in MPR deficient cells, and analysis of its multifunctional nature such as Tg and other ligands binding, would further provide evidence on the phylogenetic conservation of the multifunctional nature of the MPR 300 protein throughout vertebrates, which is the future direction of work in our laboratory.