P-Type Lectins: Cation-Dependent Mannose-6-Phosphate Receptor

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In eukaryotic cells, post-translational modification of secreted proteins and intracellular protein transport between organelles are ubiquitous features. One of the most studied systems is the *N*-linked glycosylation pathway in the synthesis of secreted glycoproteins (Schrag et al. 2003). The *N*-linked glycoproteins are subjected to diverse modifications and are transported through ER and Golgi apparatus to their final destinations in- and outside the cell. Incorporation of cargo glycoproteins into transport vesicles is mediated by transmembrane cargo receptors, which have been identified as intracellular lectins. For example, mannose 6-phosphate receptors (Ghosh et al. 2003) function as a cargo receptor for lysosomal proteins in the *trans*-Golgi network, whereas ERGIC-53 (Zhang et al. 2003) and its yeast orthologs Emp46/47p (Sato and Nakano 2003) are transport lectins for glycoproteins that are transported out of ER.

3.1 The Biosynthetic/Secretory/Endosomal Pathways

In eucaryotic cells, proteins destined for secretion are first inserted into ER and then transported by a process of vesicle budding and fusion, through the Golgi complex and then to the cell surface. Various compartments that comprise this secretory pathway, despite being interconnected by vesicular traffic, differ in their lipid and protein composition. The maintenance of these differences requires that the incorporation of molecules into vesicles is a selective process, and that vesicles are directed to specific target membranes. Much effort has been directed in recent years in understanding these processes, and the ways in which they are integrated to produce organelles of characteristic size, morphology and composition.

3.1.1 Organization of Secretory Pathway

The early secretoty pathway (ESP) is defined as sequential compartments comprising the cisternal/tubular ER, pre-Golgi intermediates [also referred to as vesicular tubular clusters (VTCs) or ER-Golgi intermediates (ERGIC)] and the cis-Golgi network (CGN), the Golgi stacks and trans-Golgi network (TGN) as a final sorting station. The basic function of this pathway includes transport of proteins destined for secretion from ER to Golgi and further to the plasma membrane (PM) (Fig. 3.1). Synthesized material can also be recycled to ER from the Golgi. The compartments are connected with each other by vesicular traffic which mediates the transport of cargo between different organelles and also controls the composition and homeostasis of the structures (Rothman and Wieland 1996). The ESP transports biosynthetic material from ER to Golgi complex. Lipids, proteins and carbohydrates are modified and transported through Golgi to TGN in which they are sorted and packed into vesicles for further transport to various destinations. At TGN, specific sorting signals in the cargo molecules and the cellular sorting machineries are responsible for directing the cargo either to the PM, to regulated secretory granules, or to the endosomal/lysosomal system (Le Borgne and Hoflack 1998). The bulk flow secretory pathway operates in all cells and it leads to a continuous unregulated secretion or transport to the PM. Some specialized cells also possess a distinct regulated secretory pathway in which certain specific proteins are secreted to extracellular space in response to external signal(s). In general, the secretory pathway provides a framework by which proteins undergo a series of posttranslational modifications including proteolytic processing, folding and glycosylation (Storrie et al. 2000).

3.1.1.1 Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a eukaryotic organelle that forms an interconnected network of tubules, vesicles, and cisternae within cells. The general structure of the ER is an extensive membrane network of cisternae (sac-like structures) held together by the cytoskeleton. The phospholipid membrane encloses a space, the cisternal space (or lumen), which is continuous with the perinuclear space but separate from

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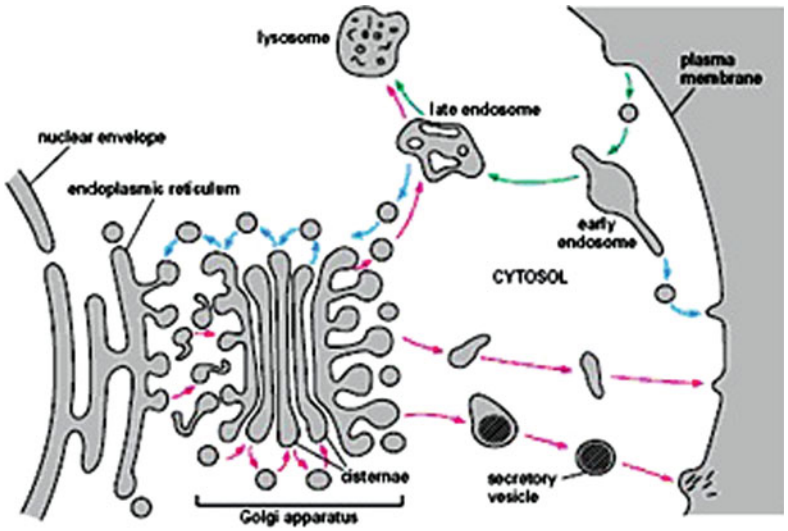

Fig. 3.1 A schematic representation of the different vesicle transport pathways originating from the *trans*-Golgi network (Reprinted by permission from from Le Borgne and Hoflack 1998 © Elsevier). *Arrows* indicate transport steps that are known to occur, but not discussed in detail in this article

the cytosol. The functions of the endoplasmic reticulum vary greatly depending on the exact type of endoplasmic reticulum and the type of cell in which it resides. The ER is the entry station for all proteins of the synthetic/secretory pathway and consists of nuclear envelope, rough ER (rER), smooth ER (sER), transitional ER (tER), and intermediate compartment (IC) (Marie et al. 2009; Lippincott-Schwartz et al. 2000). Rough endoplasmic reticula synthesize proteins, while smooth endoplasmic reticula synthesize lipids and steroids, metabolize carbohydrates and steroids, and regulate calcium concentration, drug metabolism, and attachment of receptors on cell membrane proteins. Different subcompartments of ER have characteristic biochemical and physiological properties and they serve specific subcellular functions. Structurally ER is seen as a three-dimensional, reticular network of continuous tubules and sheets creating the largest membranous organelle of the cell. Functionally ER is responsible for the synthesis and processing of secreted proteins, membrane proteins and organelle resident proteins. The ER is also part of a protein sorting pathway and seen as a compartment that participates in the assembly, sorting, and degradation of proteins as well as in the regulation of intracellular calcium concentration (Harter and Wieland 1996). It is, in essence, the transportation system of the eukaryotic cell. From ER, properly folded and assembled proteins are further transported via IC to the Golgi complex by specific carrier vesicles which bud on the ER and move to cis-Golgi membranes. Folded proteins may remain in the ER if it is their home compartment or else they are transported to the secretory pathway. COPII, a coat complex which forms a main structure of transport vesicles, is responsible for forward transport of cargo from the ER to Golgi complex. COPI vesicles in their turn carry cargo between Golgi and ER and in intra-Golgi transport (Barlowe et al. 1994; Letourneur et al. 1994; Schekman and Orci 1996). Sorting of synthesized proteins at the ER occurs by selective incorporation of secretory and membrane proteins into vesicles that bud from the ER (Pelham 1996; Wieland and Harter 1999). The majority of ER resident proteins are retained in the ER through a specific carboxy terminal retention motif. This motif is composed of four amino acids at the end of the protein sequence. The most common retention sequence is KDEL (*lys-asp-glu-leu*). However, variation on KDEL does occur and other sequences can also give rise to ER retention. There are three KDEL receptors in mammalian cells, and they have a very high degree of sequence identity. The functional differences between these receptors remain to be established. The lumenal soluble proteins of the ER carry a specific carboxyterminal KDEL signal which prevents the secretion of these proteins. Those ER-resident KDEL proteins which have escaped the ER, are recycled back from the Golgi to ER by COPI vesicles (Poussu 2001). Secretory proteins, mostly glycoproteins, are moved across the ER membrane. Proteins that are transported by the ER and from there throughout the cell are marked with an address tag called a signal sequence. The N-terminus (one end) of a polypeptide chain contains a few amino acids that work as an address tag, which are removed when the polypeptide reaches its destination. Proteins that are destined for places outside the ER are packed into transport vesicles and moved along the cytoskeleton toward their destination.

3.1.1.2 Golgi Complex

The Golgi is a stack of polarized tubular/saccular compartments with a defined cis- to trans content reflecting the presence of specialized processing enzymes that extensively modify newly synthesized proteins. The membrane-bound stacks are known as cisternae (singular: *cisterna*). Between four and eight stacks are usually present, although, in some protists as many as 60 have been observed. Each cisterna comprises a flattened membrane disk, and carries Golgi enzymes to help or to modify cargo proteins that travel through them. They are found in both plant and animal cells and the overall morphology of the ER-Golgi system can vary between cell types such as in budding to higher eukaryotes. The Golgi has earlier been viewed as a static station for the processing of secretory material, but now it seems that Golgi undergoes continuous remodeling. Traditionally, Golgi has been viewed as a series of stable compartments, named the *cis*-, medial- and *trans*-Golgi, as well as TGN (Glick 2000). The *trans*- face of the *trans*-Golgi network is the face from which vesicles leave the Golgi. These vesicles then proceed to later compartments such as the cell membrane, secretory vesicles or late endosomes. New *cisternae* form at the *cis*-Golgi network. The *cis*- and *trans*-Golgi networks are thought to be specialised *cisternae* leading in and out of the Golgi apparatus.

The Golgi apparatus is integral in modifying, sorting, and packaging macromolecules for cell secretion (exocytosis) or use within the cell. The Golgi apparatus plays an important role at the crossroads of the secretory pathway. It receives freshly synthesized proteins and lipids from the ER, modifies them, and then distributes cargo to various destinations. Proteins coming from ER to Golgi enter the organ on its tubulovesicular cis-face, travel across the stacks, and leave the Golgi on its trans-face. Cis-Golgi network not only receives the material from the ER but is also involved in sorting and recycling of lipids and proteins to the ER. On the way through the Golgi, newly synthesized glycoproteins are subjected to several posttranslational modifications such as ordered remodeling of their N-linked oligosaccharide side chains and biosynthesis of O-linked glycans. To effect such modifications, the Golgi complex is organized as polarized stacks of flattened cisternae enriched in transmembrane processing enzymes. To be able to send cargo even long distances through the cytoplasm, the Golgi complex is closely associated with the cytoskeleton. It is situated around the microtubule organizing center and is surrounded by actin cytoskeleton and actin-binding proteins (Holleran and Holzbaur 1998).

Enzymes within the cisternae are able to modify the proteins by addition of carbohydrates (glycosylation) and phosphates (phosphorylation). For example, the Golgi apparatus adds a mannose-6-phosphate label to proteins destined for lysosomes. The Golgi plays an important role in the synthesis of proteoglycans, which are molecules present in the extracellular matrix of animals. It is also a major site of carbohydrate synthesis. This includes the production of glycosaminoglycans (GAGs), long unbranched polysaccharides which the Golgi then attaches to a protein synthesised in the endoplasmic reticulum to form proteoglycans. Enzymes in the Golgi polymerize several of these GAGs via a xylose link onto the core protein. Another task of the Golgi involves the sulfation of certain molecules passing through its lumen via sulphotranferases that gain their sulphur molecule from a donor called PAPs. This process occurs on the GAGs of proteoglycans as well as on the core protein. The level of sulfation is very important to the proteoglycans' signalling abilities as well as giving the proteoglycan its overall negative charge.

3.1.1.3 Trans-Golgi Network

The trans-Golgi network is the site of the sorting and final exit of cargo from the Golgi. It refers to the trans-side of the Golgi and structurally it is seen as a sacculotubular network. The structure and the size of TGN varies remarkably from one cell type to another: in cells with a low number of secretory granules but with an extensive lysosomal system, TGN is massive, while secretory cells showing small or large secretory granules typically possess small TGN or even lack it (Clermont et al. 1995). Newly synthesized proteins traverse the Golgi stack until they reach the TGN. The trans-Golgi network sorts the proteins into several types of vesicles. Clathrin-coated vesicles carry certain proteins to lysosomes. At TGN, cargo molecules are sequestered into coated vesicles and directed to their correct destinations. For example, proteins with specific recognition signals are packed into clathrin-coated vescicles (CCVs) and transported to endosomal/lysosomal system in a selective pathway (Marks et al. 1997). Proteins carrying specific sorting signals are targeted and transported to plasma membrane through a so-called constitutive pathway (Pearse and Robinson 1990). In specialized cells producing large quantities of particular products in response to extracellular stimuli (e.g. hormonal or neural stimuli), there exists another secretion pathway leading to cell surface called the regulated secretory pathway (Traub and Kornfeld 1997). Other proteins are packaged into secretory vesicles for immediate delivery to the cell surface. Still other proteins are packaged into secretory granules, which undergo regulated secretion in response to specific signals. The sorting function of the Golgi apparatus allows the various organelles to grow while maintaining their distinct identities.

3.1.1.4 ER-Golgi Intermediate Compartment (ERGIC)

Protein traffic moving from ER to Golgi complex in mammalian cells passes through the tubulovesicular membrane clusters of the ERGIC, the marker of which is the lectin

ERGIC-53. Because the functional borders of the intermediate compartment (IC) are not well defined, the spatial map of the transport machineries operating between the ER and the Golgi apparatus remains incomplete (Fig. 3.1). However, studies showed that the IC consists of interconnected vacuolar and tubular parts with specific roles in pre-Golgi trafficking. The identification of ERGIC-53 has added to the complexity of the exocytic pathway of higher eukaryotic cells. Ffractional analysis of the ERGIC from Vero cells suggested that in the secretory pathway of Vero cells O-glycan initiation and sphingomyelin as well as glucosylceramide synthesis mainly occur beyond the ERGIC in the Golgi apparatus (Schweizer et al. 1994; Appenzeller-Herzog and Hauri 2006). Marie et al. (2009) provided novel insight into the compartmental organization of the secretory pathway and Golgi biogenesis, in addition to a direct functional connection between the IC and the endosomal system, which evidently contributes to unconventional transport of the cystic fibrosis transmembrane conductance regulator to the cell surface. The ERGIC defined by ERGIC-53 also participates in the maturation of (or is target for) several viruses such as corona virus, cytomegalovirus, flavivirus, poliovirus, Uukuniemi virus, and vaccinia virus. Understanding the targeting of viruses and viral proteins to the ERGIC could lead to development of general approaches for viral interference. Further analysis of the ERGIC-53 as a marker protein should provide novel results about the mechanisms controlling traffic in the secretory pathway (Chap. 7).

3.1.1.5 Protein Coats

Coatomer Protein Complex II (COPII) and COPI

Membrane traffic between the ER and the Golgi complex is regulated by two vesicular coat complexes, called coatomer protein complex II (COPII) and COPI. In addition, cells contain numerous clathrin/adaptor complexes—with each coat budding vesicles from a discrete subcellular location. COPII has been implicated in the selective packaging of anterograde cargo into coated transport vesicles budding from ER. In higher eukaryotes, transport from the ER is initiated by COPII mediated budding of vesicular carriers, but this is restricted to specialized, long-lived subdomains of the ER, the ER-exit sites (ERES). ERGIC clusters containing ERGIC-53 are close to but clearly distinct from ERES and delineate the subsequent stage in transport to the Golgi. The ERES are thought to generate transit vesicles and pleiomorphic tubular carriers through the activity of COPII coat machinery to yield pre-Golgi intermediates. In mammalian cells, these vesicles coalesce to form tubulo-vesicular transport complexes (TCs), which shuttle anterograde cargo from the ER to Golgi complex.

In contrast to COPII, COPI-coated vesicles are proposed to mediate recycling of proteins from the Golgi complex to the ER (David et al. 1999). The binding of COPI to COPII-coated TCs, however, has led to the proposal that COPI binds to TCs and specifically packages recycling proteins into retrograde vesicles for return to the ER. Observations, consistent with biochemical data, suggest a role for COPI within TCs *en route* to the Golgi complex. By sequestering retrograde cargo in the anterograde-directed TCs, COPI couples the sorting of ER recycling proteins to the transport of anterograde cargo (David et al. 1999; Rowe et al. 1996).

As observed, COPII-coated vesicles form on the ER to transport newly synthesized cargo to Golgi complex. Three proteins—Sec23/24, Sec13/31, and the ARF-family GTPase Sar1—are sufficient to bud ~60-nm COPII vesicles from native ER membranes and from synthetic liposomes. The COPII coat components coordinate to create a vesicle by locally generating membrane curvature and populating the incipient bud with the appropriate cargo (Lee and Miller 2007; Wiseman et al. 2007). COPII budding is initiated by the activation of Sar1 to its GTP-bound form, causing it to translocate to the membrane and embed an N-terminal α-1 helix in the bilayer (Fath et al. 2007).

Sequential Mode of Action for COPII and COPI

Exocytic transport from the ER to the Golgi complex has been visualized in living cells using a chimera of the temperature-sensitive glycoprotein of vesicular stomatitis virus and green fluorescent protein (ts-G-GFP[ct]). Upon shifting to permissive temperature, ts-G-GFP(ct) concentrates into COPII-positive structures close to ER, which then build up to form an intermediate compartment or transport complex, containing ERGIC-53 and the KDEL receptor, where COPII is replaced by COPI. These structures appear heterogenous and move in a microtubule-dependent manner toward the Golgi complex. These results suggest a sequential mode of COPII and COPI action and indicate that the transport complexes are ER-to-Golgi transport intermediates from which COPI may be involved in recycling material to the ER (Scales et al. 2000).

3.1.1.6 Clathrin Coated Vesicles (CCV)

Clathrin coats contain both clathrin and clathrin adaptor proteins. While clathrin acts as the scaffold, the clathrin adaptors bind to protein and lipid cargo. Specific cargos are recruited into clathrin-coated vesicles with the aid of CLASP proteins (clathrin-associated sorting proteins), such as ARH and Dab2. Clathrin-associated protein complexes are believed to interact with the cytoplasmic tails of membrane proteins, leading to their selection and concentration. At least 20 clathrin adaptors have been identified, which share a common design composed of a compact domain plus a long unstructured region that binds the clathrin beta-propeller. The two major types of clathrin adaptor complexes are: heterotetrameric adaptor protein

(AP) complexes, and the monomeric GGA (Golgi-localising, γ-adaptin ear domain homology, ARF-binding proteins) adaptors. Whereas clathrin heavy chain provides the structural backbone of the clathrin coat, it was suggested that clathrin light chains (CLCs) are not required for clathrin-mediated endocytosis but are critical for clathrin-mediated trafficking between the TGN and the endosomal system. In CLC-deficient mice CI-MPRs cluster near the TGN leading to a delay in processing of the lysosomal cathepsin D. In mammalian cells CLCs function in intracellular membrane trafficking by acting as recruitment proteins for huntingtin-interacting protein 1-related (HIP1R), enabling HIP1R to regulate actin assembly on clathrin-coated structures (Poupon et al. 2008).

3.1.1.7 Adaptor Protein Complexes

Adaptor protein (AP) complexes are found in coated vesicles and clathrin-coated pits. AP complexes connect cargo proteins and lipids to clathrin at vesicle budding sites, as well as binding accessory proteins that regulate coat assembly and disassembly. There are different AP complexes in mammals. AP1 is responsible for the transport of lysosomal hydrolases between the TGN and endosomes. AP2 associates with the plasma membrane and is responsible for endocytosis. The AP-1 and AP-2 complexes are the most abundant adaptors in CCVs, but clathrin-mediated trafficking can still occur in the absence of any detectable AP-1 or AP-2. AP3 is responsible for protein trafficking to lysosomes and other related organelles. AP4 is less well characterised. AP-4 is localized mainly in the Golgi complex, as well as on endosomes and transport vesicles. Mammary epithelial cells contain an unexpectedly high quantity of clathrin coated vesicles. Analysis of CCV adaptor composition showed that approximately 5–10% of total APs consist of AP-2 in mammary gland CCV whereas it represents approximately 70% of the total APs from bovine brain CCV. Relatively high quantities of furin and CI-MPR were detected in mammary CCV. AP-1 and the CI-MPR were localized in Golgi-associated vesicles and on the membrane of secretory vesicles. CCV in lactating mammary epithelial cells are involved in the transcytotic pathway, in sorting at the TGN and in the biogenesis of casein-containing secretory vesicles (Pauloin et al. 1999).

3.1.1.8 Lysosomes and Role of Mannose 6-Phosphate

The main function of lysosomes in the cell is the degradation of internalized material by lysosomal enzymes, the acid hydrolases. These enzymes are synthesized in the ER and during their maturation in the Golgi apparatus they acquire the mannose 6-phosphate (M6P) recognition marker. Most of these soluble hydrolases are transported to lysosomes through specific M6P receptors. The receptor-

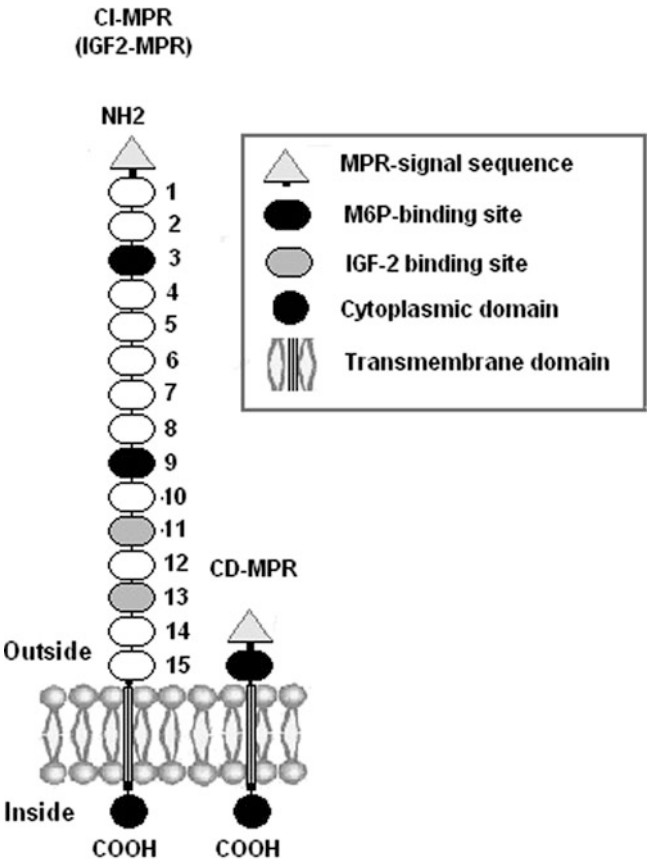

Fig. 3.2 Schematic diagram of the full-length MPRs. The MPRs are type I transmembrane glycoproteins that consist of an N-terminal (NH2) signal sequence, an extracytoplasmic region, a single transmembrane domain, and a C-terminal (COOH) cytoplasmic tail (Hancock et al. 2002)

enzyme complexes segregated in TGN are transferred to the lysosomal compartments *via* the secretory pathway through clathrin-coated vesicles.In mammalian cells, the targeting of newly synthesized hydrolases, as well as others ligands with M6P residues on their N-linked oligosaccharides, to the lysosomes depends on their recognition by two specific M6P receptors at the TGN: the cation-dependent M6P receptor (CD-MPR) and the cation-independent M6P receptor (CI-MPR), also called mannose 6-phosphate/insulin-like growth factor II receptor in eutherian mammals, in which it binds IGF-II (Kornfeld 1992; Varki and Kornfeld 2009) (Fig. 3.2). These two receptors (M6PRs) are considered sorting receptors because of their routing function. In the pre-lysosomal compartment, acidity induces release of enzymes from both MPR, which are then recycled into the Golgi apparatus. However, the CI-MPR, which participates to this cellular routing, is also anchored to the cell surface membrane and can internalize extracellular ligands (Kornfeld 1992; Munier-Lehmann et al. 1996; Dahms 1996). Thus, the pool of lysosomal acid hydrolases comes both from in situ synthesis, which involves the two

M6P receptors, and from endocytosis through the CI-MPR (Ni et al. 2006; Nykjaer et al. 1998). It is now well accepted that the TGN is the major site where proteins are sorted from the biosynthetic pathway for efficient delivery to endosomes. There, the MPRs and their bound ligands are segregated into nascent clathrin-coated vesicles, probably together with other transmembrane proteins destined to the endosomes/lysosomes. After budding and uncoating, these Golgi-derived vesicles fuse with endosomal compartments where the MPRs discharge their bound ligands. While the soluble lysosomal enzymes are directed toward the lysosomes, the MPRs recycle back to the TGN (Duncan and Kornfeld 1988) or to the cell surface where they are found in small amounts at steady state. At the plasma membrane, the MPRs undergo endocytosis via clathrin-coated pits like many other cell surface receptors (Fig. 3.1).

3.2 P-Type Lectin Family: The Mannose 6-Phosphate Receptors

The best-characterized function of two MPRs, CI-MPR and CD-MPR of 46 kDa and 300 kDa respectively, is their ability to direct the delivery of approximately 60 different newly synthesized soluble lysosomal enzymes bearing M6P on their N-linked oligosaccharides to lysosome. The CI-MPR is a multifunctional protein which binds at the cell surface to two distinct classes of ligands, the M6P bearing proteins and IGF-II. In addition to its intracellular role in lysosome biogenesis, the CI-MPR, but not the CD-MPR, participates in a number of other biological processes by interacting with various molecules at the cell surface. Though, the major function of CI-MPR is to bind and transport M6P-enzymes to lysosomes, but it can also modulate the activity of a variety of extracellular M6P-glycoproteins (i.e., latent TGFβ precursor, urokinase-type plasminogen activator receptor, Granzyme B, growth factors, Herpes virus). The synthesis and potential use of high affinity M6P analogues able to target this receptor have been described. Several M6P analogues with phosphonate, carboxylate or malonate groups display a higher affinity and a stronger stability in human serum than M6P itself. These derivatives can be used to favour the delivery of specific therapeutic compounds to lysosomes, notably in enzyme replacement therapies of lysosomal diseases or in neoplastic drug targeting (Gary-Bobo et al. 2007). In addition, their potential applications in preventing clinical disorders, which are associated with the activities of other M6P-proteins involved in wound healing, cell growth or viral infection, have been discussed.The list of extracellular ligands recognized by this multifunctional receptor has grown to include a diverse spectrum of M6P-containing proteins as well as several non-M6P-containing ligands. Structural studies have shown how these two receptors use related, but yet distinct, approaches in the recognition of phosphomannosyl residues (Dahms et al. 2008).

The two receptors, which share sequence similarities, constitute the P-type family of animal lectins. The CD-MPR (46 kDa) and the CI-MPR (300 kDa) are ubiquitously expressed throughout the animal kingdom and are distinguished from all other lectins by their ability to recognize phosphorylated mannose residues (Fig. 3.2). The P-type lectins play an essential role in the generation of functional lysosomes within the cells of higher eukaryotes by directing newly synthesized lysosomal enzymes bearing M6P signal to lysosomes. At the cell surface, the IGF2R/CI-MPR also binds to the nonglycosylated polypeptide hormone, IGF-II, targeting this potent mitogenic factor for degradation in lysosomes. The two MPRs have overlapping function in intracellular targeting of newly synthesized lysosomal proteins, but both are required for efficient targeting. Their main function is to transport lysosomal enzymes from TGN to the pre-lysosomal compartment. MPRs are conserved in the vertebrates from fish to mammals and show non-identical distribution among sub-cellular fractions in liver (Dahms and Hancock 2002; Messner et al. 1989; Messner 1993). Although much has been learned about the MPRs, it is unclear how these receptors interact with the highly diverse population of lysosomal enzymes. It is known that the terminal M6P is essential for receptor binding. Mannose receptor-enriched membranes of liver sinusoidal cells contain significant levels of CD-MPR, but not the CI-MPR. Both CD-MPRs and CI-MPRs bind their M6P-tagged cargo in the lumen of the Golgi apparatus in the cell. The CD-MPR shows greatly enhanced binding to M6P in the presence of divalent cations, such as manganese. M6P-containing proteins can be purified on immobilized MPRs. The sequences of zebrafish (*Danio rerio*) CD-MPR and CI-MPR (Nolan et al. 2006) indicate that targeting of lysosomal enzymes by MPRs is an ancient pathway in vertebrate cell biology. Yadavalli and Nadimpalli (2008) reported putative MPR receptors from starfish. Structural comparison of starfish receptor sequences with other vertebrate receptors gave structural homology with the vertebrate MPR-46 protein. The expressed protein in $mpr^{-/-}$ mouse embryonic fibroblast cells efficiently sorts lysosomal enzymes within the cells establishing a functional role for this protein (Yadavalli and Nadimpalli 2008). The insect cell line *Sf9* infected with a recombinant baculovirus containing the gene for human prorenin, cultured in presence of ^{3}H-mannose do not synthesize high-mannose-type oligosaccharides containing M6P, and consequently it appears unlikely that these cells utilize the MPR mediated pathway for targeting of lysosomal enzymes (Aeed and Elhammer 1994). Recently P-type CRD-like domains have been found in proteins with different architectures to the MPRs, and have been termed MPR homology (MRH) domains. Some of the MRH domains in

non-MPR proteins are known to have sugar-binding activity, and glycan recognition may be a general function of the MRH domain.

3.2.1 Fibroblasts MPRs

The turnover of the phosphomannosyl receptor in fibroblasts is very slow, in contrast with its rate of internalization in endocytosis, and that its rate of degradation is not greatly altered by a variety of agents that affect lysosomal protein turnover and/or receptor-mediated endocytosis (Creek and Sly 1983). The MPR, on the surface of fibroblasts, accounts for the intracellular transport of newly synthesized enzymes to the lysosome. Fibroblasts MPRs internalized oligosaccharides of known specific activity bearing a single phosphate in monoester linkage with K_{uptake} of 3.2×10^{-7} M, whereas oligosaccha- rides bearing two phosphates in monoester linkage were internalized with a K_{uptake} of 3.9×10^{-8} M (Natowicz et al. 1983).

3.2.2 MPRs in Liver

During perinatal development, the CI-MPR expression decreases progressively from 18-day fetuses to adults, whereas the CD-MPR showed a transient decrease in newborn and at the fifth day after birth in rats. Both receptors localize to hepatocytes at all ages and, additionally, the CD-MPR was reactive in megakaryocytes at early stages. In adult rat liver, CIMPR is detected intensely in hepatocytes and weakly in sinusoidal Kupffer cells and interstitial cells in Glisson's capsule. A high level of expression of CI-MPR mRNAs in hepatocytes and of CD-MPR mRNA in Kupffer cells was detected by in situ hybridization. Differential changes during perinatal development and adults suggest that two MPRs play distinct roles during organ maturation (Romano et al. 2006; Waguri et al. 2001). It was found that the activity of glycosidases changes during development, reaching a peak at the tenth day after birth and correlated with the expression and binding properties of CD-MPR. It was suggested that lysosome maturation in rat liver occurs around tenth day after birth, and that the CD-MPR may participate in that event. In hepatocytes of MPR-deficient neonatal mice, lysosomal storage occurs when both MPRs are lacking, whereas deficiency of CI-MPR only has no effect on the ultrastructure of the lysosomal system (Schellens et al. 2003). A biochemical comparison between autophagosomes and amphisomes from rat liver showed that the amphisomes were enriched in early endosome markers [the asialoglycoprotein receptor and the early endosome-associated protein as well as in a late endosome marker (CI-MPR)]. Amphisomes would thus seem to be capable of receiving inputs both from early and late endosomes (Berg et al. 1998). In human HepG2 and BHK cells, the two receptors were identified at the same sites: the trans-Golgi reticulum (TGR), endosomes, electron-dense cytoplasmic vesicles, and the plasma membrane. It was suggested that the two MPRs exit TGR via same coated vesicles. However, on arrival in the endosomes CD-MPR is more rapidly than CI-MPR segregated into associated tubules and vesicles (ATV) which probably are destined to recycle MPRs to TGR (Klumperman et al. 1993).

3.2.3 MPRs in CNS

The two related but distinct MPRs have been localized in neurons of mouse CNS, with more intense labeling in the medial septal nucleus, the nucleus of the Broca's diagonal band, layers IV-VI of the cerebral neocortex, layers II-III of the entorhinal cortex, the habenular nucleus, the median eminence, several nuclei and structures of the brainstem, the Purkinje cell layer of the cerebellum, and in the ventral horn of the spinal cord. Although intense reactivities of both MPRs were observed in the same groups of neurons in the same regions, the spatial differences in immunoreactive intensity for CI-MPR were greater, particularly in the telencephalon such as the basal forebrain and cerebral cortex, than those for CD-MPR. While CD-MPR is ubiquitously necessary for the general function of neurons, the CI-MPR is selectively necessary for certain region- and neurotransmitter-specific functions of neurons (Konishi et al. 2005). In rat brain during perinatal development, the expression of CI-MPR decreases progressively from fetuses to adults, where as CD-MPR increases around the tenth day of birth, and maintained there after. This shows that the two receptors play a different role in rat brain during perinatal development; CD-MPR being mostly involved in lysosome maturation (Romano et al. 2005). Given the critical role of endosomal-lysosomal (EL) system in the clearance of abnormal proteins, it is likely that the increase in the CI-MPR and components of the EL system in surviving neurons after 192-IgG-saporin represents an adaptive mechanism to restore the metabolic/structural abnormalities induced by the loss of cholin-ergic neurons (Hawkes et al. 2006).

A lysosomal enzyme binding receptor protein from monkey brain shows protein kinase activity and undergoes phosphorylation on serine and tyrosine residues. The lysosomal enzyme fucosidase and M6P, which are ligands for the receptor, stimulated the activity of protein phosphatase associated with the receptor protein. A phosphorylation/dephosphorylation mechanism may be operative in the ligand binding and functions of the receptor (Panneerselvam and Balasubramanian 1993).

The prion encephalopathies are characterized by accumulation of the abnormal form PrPsc of a normal host gene

product PrPc in the brain. In search of the mechanism and site of formation of PrPsc from PrPc in ME7 scrapie-infected mouse brain, it was found that proteinase K-resistant PrPsc is enriched in subcellular structures which contain CI-MPR, ubiquitin-protein conjugates, β-glucuronidase, and cathepsin B, termed late endosome-like organelles. The organelles may act as chambers for the conversion of PrPc into infectious PrPsc in murine model of scrapie (Arnold et al. 1995).

In neuroendocrine cells sorting of proteins from immature secretory granules (ISGs) occurs during maturation and is achieved by CCV containing AP-1. The MPRs are detected in ISGs of PC12 cells and more than 80% of the ISGs contained furin. Fifty percentage at most of the ISGs contained CI-MPR. Dittié et al. (1999) suggested the presence of two populations of ISGs: those that have both MPRs and furin, and those which contain only furin. It was shown that binding of adapter protein-1 (AP-1) requires casein kinase II phosphorylation of CI-MPRfusion protein, and in particular phosphorylation of Ser-2474.

The β-amyloid deposits in the brains of all patients of Alzheimer's disease (AD). Stephens and Austen (1996) defined major location of β-amyloid precursor protein fragments possessing the Asp-1 N-terminus of β-amyloid as the TGN or late endosome on the basis of colocalisation with a mAb to the CI-MPR. The co-localisation suggested that the p13 fragment and MPR are trafficked by alternative pathways from TGN. Hawkes and Kar (2004) delineated the role of the CI-MPR in the CNS, including its distribution, possible importance as well as its implications in neurodegenerative disorders such as AD.

3.2.4 CI-MPR in Bone Cells

The osteoclast is a polarized cell which secretes large amounts of newly synthesized lysosomal enzymes into an apical extracellular lacuna where bone resorption takes place. Osteoclast expresses large amounts of immunoreactive CI-MPR, despite the fact that most of the lysosomal enzymes it synthesizes are secreted. In osteoclast, M6P receptors are involved in the vectorial transport and targeting of newly synthesized lysosomal enzymes, presumably via a constitutive pathway, to the apical membrane where they are secreted into the bone-resorbing compartment. This mechanism could insure polarized secretion of lysosomal enzymes into the bone-resorbing lacuna (Baron 1989). The rapid inhibition of bone resorption by calcitonin involves the vesicular translocation of the apical membranes and the rapid arrest in the synthesis and secretion of lysosomal enzymes in osteoclasts (Baron et al. 1990). IGF2R/CI-MPR is present in rat calvarial osteoblasts. Osteoblasts bind IGF-II with high affinity ($K_D \sim 2.0$ nM). The osteoblastic Ca^{2+} response to IGF-II is caused by an intracellular release of Ca^{2+} which is mediated by the IGF-II/CI-MPR (Martinez et al. 1995). The phosphorylated monosaccharide, M6P stimulates alkaline phosphatase produced by osteoblasts. Glucose-6-phosphate and fructose-1-phosphate also stimulated osteoblast alkaline phosphatase production, but not to the same extent as M6P. Since, the stimulatory effect of M6P is similar to that of IGF-II, it supports similar mechanism for signal transduction for both IGF-II and M6P (Ishibe et al. 1991).

Secretory ameloblasts possesses strong immunoreactivity for MPR in the supranuclear Golgi region and in the cytoplasm between the Golgi region and the distal junctional complexes, where as cathepsin B immunoreactivity was mainly seen in the distal portion of Tomes' process, which was unreactive for MPR immunogenicity. Since MPR and lysosomal enzymes were also detected on the ruffled border of osteoclasts adjacent to alveolar bone, report provides strong evidence for a similarity between the maturation process in enamel, as mediated by ameloblasts, and bone resorption mediated by osteoclasts (Al Kawas et al. 1996).

3.2.5 Thyroid Follicle Cells

Thyroglobulin (Tg), the major secretory product of thyrocytes, is the macromolecular precursor of thyroid hormones. The Tg has been shown to be phosphorylated and to carry M6P signal in terminal position. In porcine thyroid follicle cells, the CI-MPR is primarily located in elements of the endocytic pathway such as coated pits and endosomes. This localization of the CI-MPR in thyrocytes differs from the receptor sites in other cell types by the rare occurrence of CI-MPR in cisternae of the Golgi complex. The CI-MPR in thyrocytes might be unable to bind and to convey Tg efficiently. The receptor is, however, a binding site for Tg at the apical plasma membrane and may, therefore, be involved in the binding of Tg and its transfer from the follicle lumen to lysosomes (Lemansky and Herzog 1992; Scheel and Herzog 1989). Using antibodies against Tg and CI-MPR, Kostrouch et al. (1991) suggested three types of endocytic structures: those slightly positive for MPR and ArS-A, those strongly positive for both markers, and those only positive for ArS-A. These compartments exhibited the properties of early endosomes (EE), late endosomes (LE), and lysosomes (L), respectively. The data indicate that internalized Tg molecules are transported to EE and then transferred from EE to LE.

3.2.6 Testis and Sperm

M6P receptors have been isolated from germ cells and Sertoli cells present in testes. Isolated mouse pachytene

spermatocytes and round spermatids synthesize predominantly the 46 kDa CD-MPR and only low levels of the 270 kDa CI-MPR. In contrast, Sertoli cells synthesized substantial amounts of the CI-MPR, but little of CD-MPR. Like germ cells, Sertoli cells in primary culture endocytosed ^{125}I-M6P-bearing ligands at levels that were about 10% of the endocytic activity measured for 3T3 fibroblasts. This indicates that both spermatogenic and Sertoli cells have surface MPRs capable of mediating endocytosis (O'Brien et al. 1989, 1993). Tsuruta and O'Brien (1995) and Tsuruta et al. (2000) provided evidence that IGF-II/Cl-MPR ligands secreted by Sertoli cells can modulate gene expression in spermatogenic cells and strongly suggest that they are important in the regulation of spermatogenesis. Moreno (2003) studied the dynamics of some components of the endosome/lysosome system, as a way to understand the complex membrane trafficking circuit established during spermatogenesis and suggested that the CI-MPR could be involved in membrane trafficking and/or acrosomal shaping during spermiogenesis.

A single CI-MPR transcript, approximately 10 kb in size, was present in mouse spermatogenic and Sertoli cells. Like the CI-MPR protein, its mRNA transcript was more abundant in Sertoli cells than in spermatogenic cells from adult testes. The CD-MPR was the predominant MPR synthesized by pachytene spermatocytes or round spermatids. Multiple CD-MPR transcripts were detected in these cells, including a 2.4-kb CD-MPR mRNA that was indistinguishable from CD-MPR transcripts in somatic tissues and Sertoli cells. Results suggested that alternate polyadenylation signals are used to produce multiple CD-MPR transcripts in spermatogenic cells (O'Brien et al. 1994). Low molecular weight M6P-receptors from bovine testis exhibited two isoforms with Mr of 45,000 (MPR-2A) and 41,000 (MPR-2B). These isoforms contain a common polypeptide core, but differ in their carbohydrate content (Li and Jourdian 1991). Two (pro)renin receptors have been characterized so far, the MPR and a specific receptor called (P)RR for (pro)renin receptor. Each receptor controls a different aspect of renin and prorenin metabolism. The MPR is a clearance receptor, whereas (P)RR mediates their cellular effects by activating intracellular signaling and up-regulating gene expression.

Belmonte et al. (1998) demonstratesd that α-mannosidase from rat epididymal fluid is a ligand for phosphomannosyl receptors on the sperm surface. Evidence is also presented that the CI-phosphomannosyl receptors are responsible for the interaction with alpha-mannosidase. These findings suggest a new role for extracellular transport mediated by the M6P receptor. Both MPRs undergo changes in distribution as spermatozoa passed from rete testis to cauda epididymis. CI-MPR was concentrated in the dorsal region of the head in rete testis sperm and that this labeling extended to the equatorial segment of epididymal spermatozoa. CD-MPR, however, changed from a dorsal distribution in rete testis, caput, and corpus to a double labeling on the dorsal and ventral regions in cauda spermatozoa; staining for either CI-MPR or CD-MPR increased from rete testis to epididymis. Changes in MPRs distribution may be related to a maturation process, which suggests new roles for the phosphomannosyl receptors (Belmonte et al. 2000). The targeted disruption of either MPR does not result in decreased acrosomal targeting efficiency (Chayko and Orgebin-Crist 2000).

3.2.7 MPRs During Embryogenesis

The MPR46 showed high expression at the sites of hemopoiesis and in the thymus while MPR300 was highly expressed in the cardiovascular system. Late in embryogenesis (day 17.5) a wide variety of tissues expressed the receptors, but still the expression pattern was almost non-overlapping. This unexpected spatially and temporally expression pattern points to specific functions of the two MPRs during mouse embryogenesis (Matzner et al. 1992).

3.3 Cation-Dependent Mannose 6-Phosphate Receptor

3.3.1 CD-MPR- An Overview

The cation-dependent mannose 6-phosphate receptor (or CD-MPR) is one of two proteins that bind M6P tags on acid hydrolase precursors in the Golgi apparatus that are destined for transport to the endosomal-lysosomal system. The CD-MPR recognizes the phosphomannosyl recognition marker of lysosomal enzymes. Homologues of CD-MPR are found in all eukaryotes. The CD-MPR is a type I transmembrane protein with a single transmembrane domain. The extracytoplasmic/lumenal M6P binding-domain consists of 157 amino acid residues. The bovine CD-MPR is composed of a 28-residue amino-terminal signal sequence, a 159-residue extracytoplasmic region, a 25-residue transmembrane region, and a 67-residue carboxyl-terminal cytoplasmic domain. The extracytoplasmic region of the CD-MPR contains 6 cysteine residues that are involved in the formation of three intramolecular disulfide bonds that play an essential role in the folding of the receptor (Wendland et al. 1991) (Fig. 3.2).

The CD-MPR from P388D1 macrophages lacks 215-kDa MPR. An identical protein was purified from bovine liver. The MPR binds efficiently to phosphomannosyl monoester-containing ligands in presence of MnCl2. The receptor contains both high mannose (or hybrid)- and complex-type oligosaccharide units on the basis of sensitivity to digestion with endo-beta-N-acetylglucosaminidase H and endo-β-N-acetylglucosaminidase F. The 46-kDa CD-MPR and the 215-kDa CI-MPR not only differ in their properties but are also immunologically distinct (Hoflack and Kornfeld 1985).

The receptor from human liver has a subunit molecular size of 43 kDa. It is rich in hydrophobic and charged amino acids and contains threonine at the N-terminus. The receptors from human and rat liver are antigenically related. Both are immunologically distinct from the CI-MPR of 215-kDa from human liver. The CD receptor exists in solution as a dimer or tetramer (Dahms and Hancock 2002). Modification of arginine and histidine residues reduced the binding of the receptor to immobilized ligands. Presence of M6P during modification of arginine residues protected the binding properties of the receptor, suggesting that arginine is a constituent of the M6P binding site of the receptor (Stein et al. 1987c). PC12 cells express CI-MPR, but not CD-MPR as much. The CD-MPR preferentially transports cathepsin B in PC12 cells, and cathepsins B and D participate in the regulation of PC12 cell apoptosis (Kanamori et al. 1998).

The cDNA clones encoding entire sequence of bovine 46-kDa CD-MPR, in *Xenopus laevis* oocytes results in a protein that binds specifically to phosphomannan-Sepharose and a deduced 279 amino acid sequence reveals a single polypeptide chain that contains a putative signal sequence and a transmembrane domain. The microsomal membranes containing the receptor and the location of the five potential N-linked glycosylation sites indicate that the receptor is a transmembrane protein with an extracytoplasmic amino terminus. This extracytoplasmic domain is homologous to the approximately 145 amino acid long repeating domains present in the 215-kDa CI-MPR (Dahms et al. 1987). The full-length cDNA for the goat CD-MPR46 protein was expressed in MPR deficient cells. It exhibits oligomeric nature as observed in the other species. The binding and sorting functions of the expressed protein to sort cathepsin D to lysosomes were similar to natural protein (Poupon et al. 2007). 46-kDa MPR mediates transport of endogenous but not endocytosis of exogenous lysosomal enzymes. Internalization of receptor antibodies indicated that the failure to mediate endocytosis of lysosomal enzymes is due to an inability of surface 46-kDa MPR to bind ligands rather than its exclusion from the plasma membrane or from internalization (Stein et al. 1987a, b).

3.3.2 Human CD-MPR

c-DNA clones for the human CD-MPR from a human placenta encoding the nucleotide sequence of the 2463-bp cDNA insert includes a 145-bp 5′ untranslated region, an ORF of 831 bp corresponding to 277 amino acids (Mr = 30,993), and a 1487-bp 3′ untranslated region. The deduced amino acid sequence is colinear with that determined by amino acid sequencing of the N-terminus peptide (41 residues) and nine tryptic peptides (93 additional residues). The receptor is synthesized as a precursor with a signal peptide of 20 amino acids. The hydrophobicity profile of the receptor indicates a single membrane-spanning domain, which separates an N-terminal region containing five potential N-glycosylation sites from a C-terminal region lacking N-glycosylation sites. Thus the N-terminal (Mr = 18,299) and C-terminal (Mr less than or equal to 7,648) segments of the mature receptor are assumed to be exposed to the extracytosolic and cytosolic sides of the membrane, respectively. The gene for the receptor is located on human Chromosome 12 *p13* (Ghosh et al. 2003).

The human MPR46 gene is distributed over 12 kb and divided into seven exons (110–1573 bp). All the intron/exon borders agree with the consensus sequences of splice junctions. Exon 1 codes for a 5′ untranslated sequence. The ATG initiation codon begins with the second nucleotide in exon 2. A signal sequence of 26 amino acid residues is followed by the extracytoplasmic (luminal) domain, which extends to exon 5. The transmembrane domain of the receptor spans exons 5 and 6 and the cytoplasmic domain is encoded by exons 6 and 7. The latter domain also codes for an extended 3′ untranslated sequence. The transcription-initiation site was defined by primer extension. The sequence upstream of the cap site has strong promoter activity and contains structural elements characteristic of promoters found in housekeeping genes. No correlation between the genomic organization and known protein domains of the MPR46 was apparent (Klier et al. 1991). Moreover, the sequence of about 150 amino acids within the luminal domain of MPR46, which is homologous to the 15 repeats that constitute the luminal domain of the MPR300, does not correlate with intron/exon borders. MPR46 and MPR300 have therefore diverged from a common ancestral gene before introduction of the present intron sequences.

3.3.3 Mouse CD-MPR

A cDNA clone for mouse MPR revealed a single open reading frame that codes for a protein of 278 residues. It shows an over-all amino-acid identity of 93% with the human receptor. Nine non-conservative amino-acid exchanges are found in the luminal domain, one non-conservative exchange of hydrophobic amino acids is in the transmembrane domain, while the cytoplasmic receptor tails are identical. All five potential N-glycosylation sites are conserved as well as amino acids that are important for ligand binding (Arg^{137} and His^{131}) and disulfide pairing (Cys^{32} and Cys^{78}, Cys^{132} and Cys^{167}, Cys^{145} and Cys^{179}). The absolute identity in the cytoplasmic MPR46 tail suggests the importance of this amino-acid sequence for the intracellular routing of the MPR46 (Köster et al. 1991). The 278-amino acid sequence deduced from the cDNA for the murine MPR46 shows 19 amino acid differences from that of the human MPR46, none of which are found in the 68-amino acid cytoplasmic tail. Binding of ligand to the murine MPR46 in permeabilized cells showed a pH optimum of 6.5, was completely inhibited

by M6P, and was stimulated by divalent cations. Mn^{2+} was more effective than Ca^{2+} or Mg^{2+}. Endocytosis was demonstrated at pH 6.5 and was stimulated four- to sevenfold by Mn^{2+}. In its responsiveness to divalent cations and its preference for Mn^{2+}, the murine 46MPR resembled the bovine 46MPR more than the human 46MPR. It was no more efficient than the human 46MPR in correcting the sorting defect of IGF-IIR/MPR-deficient mouse L cells (Ma et al. 1991).

3.3.3.1 Pseudogene of Mouse CD-MPR

Ludwig et al. (1992) cloned the mouse CD-MPR gene and also a very unusual processed-type CD-MPR pseudogene. Both are present at one copy per haploid genome and map to chromosomes 6 and 3, respectively. Comparison of the complete 10-kb sequence of the functional gene with the cDNA indicates that it contains seven exons. Exon 1 encodes the 5′-untranslated region of the mRNA, the others (exons 2–7) encode the luminal, transmembrane, and cytoplasmic domains of the CD-MPR. Exon 7 also contains a 1.2-kb-long 3′-untranslated region of the mRNA. A unique transcription-initiation site was determined by primer extension of mouse liver mRNA. The promoter elements in the 5′ upstream region of this site resemble those contained in genes constitutively transcribed. However, Northern blot analysis demonstrates that the CD-MPR is variably expressed in adult mouse tissues and during mouse development. The pseudogene, which is flanked by direct repeats, is almost colinear with the cDNA indicating that it presumably arose by reverse transcription of an mRNA. However, the pseudogene differs from the cDNA. It contains at its 5′ end, an additional 340-nucleotide (nt) sequence homologous to the promoter region of the functional gene. This sequence exhibits some promoter activity in vitro. Furthermore, a 24-nt insertion interrupts the region homologous to the 5′-noncoding region of the cDNA. In the functional gene, this 24-nt sequence occurs between exon 1 and 2, where it is flanked by typical consensus sequences of exon/intron boundaries. Therefore, it may represent an additional exon of the functional gene. These two features of the pseudogene suggest that expression of the CD-MPR gene may be regulated by use of different promoters and/or alternative splicing.

3.4 Structural Insights

3.4.1 N-Glycosylation Sites in CD-MPR

The bovine CD-MPR contains five potential N-linked glycosylation sites, four of which are utilized. CD-MPR mutants lacking various potential glycosylation sites showed that the presence of a single oligosaccharide chain, particularly at position 87 significantly enhanced its M6P-binding ability when compared with non-glycosylated receptors. It was suggested that N-glycosylation of the bovine CD-MPR facilitates the folding of the nascent polypeptide chain into a conformation that is conducive for intracellular transport and ligand binding (Zhang and Dahms 1993). A soluble truncated form CD-MPR encoding only the extracytoplasmic region, Stop155, and a truncated glycosylation-deficient form of the CD-MPR, Asn81/Stop155, which has been modified to contain only one N-linked glycosylation site at position 81 instead of five, were purified from baculovirus-infected High Five insect cells. The extracellular region of the CD-MPR is sufficient for high-affinity binding and that oligosaccharides at positions 31, 57, and 87 do not influence ligand binding (Marron-Terada et al. 1998). The recombinant insect-produced CD-MPR existed as a dimer in the membrane. The cytoplasmic domains of the MPRs are sufficient to determine the steady-state distribution of the full-length proteins (Dahms and Hancock 2002; Mauxion et al. 1995).

Mammalian cell lysosomal enzymes or phosphorylated oligosaccharides derived from them are endocytosed by a MPR found on the surface of fibroblasts. Studies suggest that two residues of M6P in phosphomonoester linkage but not diester linkage (PDE) are essential for a high rate of uptake. The lysosomal enzymes of the slime mold *Dictyostelium discoideum* are also recognized by the MPR on these cells; however, none of the oligosaccharides from these enzymes contain two phosphomonoesters. Instead, most contain multiple sulfate esters and two residues of M6P in an unusual PDE linkage. Further study shows that nearly all of the α-mannosidase molecules contain the oligosaccharides required for uptake, and that each tetrameric, holoenzyme molecule has sufficient carbohydrate for an average of 10 Man8GlcNAc2 oligosaccharides. Results suggest that the interactions of multiple, weakly binding oligosaccharides, especially those with 2 PDE, are important for high rate of uptake of the slime mold enzymes. The conformation of the protein may be important in orienting the oligosaccharides in a favorable position for binding to MPR (Freeze 1985).

3.4.2 3-D Structure of CD-MPR

Roberts et al. (1998) reported the three-dimensional structure of a glycosylation-deficient, yet fully functional form of the extracytoplasmic domain of the bovine CD-MPR (residues 3–154) complexed with M6P at 1.8 A resolution. The extracytoplasmic domain of the CD-MPR crystallizes as a dimer, and each monomer folds into a nine-stranded flattened beta barrel, which bears a striking resemblance to avidin (Fig. 3.3). The distance of 40 A between the two ligand-binding sites of the dimer provides a structural basis for the observed differences in binding affinity exhibited by the CD-MPR toward various lysosomal enzymes.

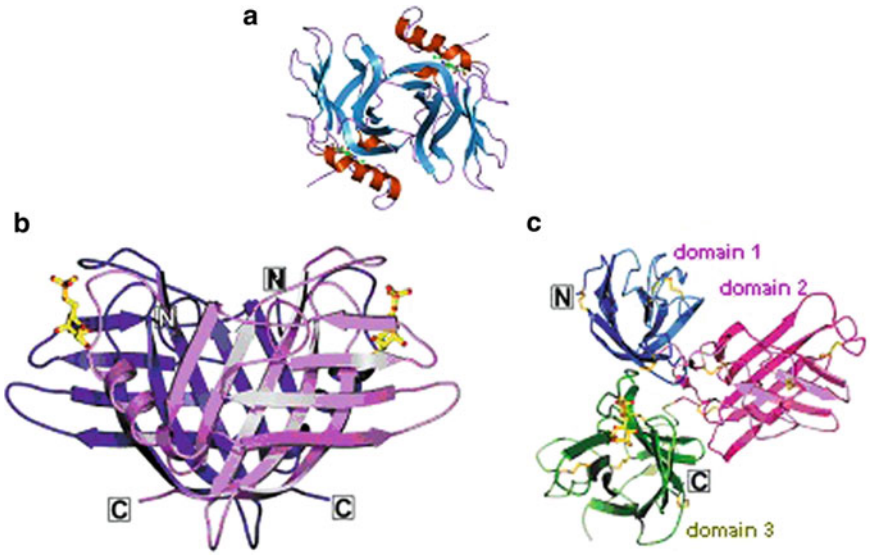

Fig. 3.3 Crystal structure of CD-MPR (46 kDa in humans) (**a**) (PDB ID: 1KEO). (**b**) Crystal structure of the extracytoplasmic region (residues 3–154) of the bovine CD-MPR in the presence of an oligosaccharide, pentamannosyl phosphate (PDB 1 C39). Note that only the terminal Man-6-P (*gold* ball-and-stick model) is shown for clarity. Both monomers (*light purple* and *dark purple*) of the CD-MPR dimer are shown in this ribbon diagram. The N-terminus (N) and C-terminus (C) are boxed. (**c**) Crystal structure of the N-terminal three domains (residues 7–432) of the bovine CI-MPR (PDB 1SZO). The N- and C-terminus of the protein encoding domain 1 (*blue*), domain 2 (*pink*), and domain 3 (*green*) are indicated. The location of Man-6-P (*gold* ball-and-stick model) is shown (Reprinted with permission from Dahms et al. 2008 © Oxford University Press)

Studies using synthetic oligosaccharides indicated that the binding site encompasses at least two sugars of the oligosaccharide. Olson et al. (1999b) reported the structure of the soluble extracytoplasmic domain of a glycosylation-deficient form of the bovine CD-MPR complexed to pentamannosyl phosphate. This construct consists of the amino-terminal 154 amino acids (excluding the signal sequence) with glutamine substituted for asparagine at positions 31, 57, 68, and 87. The binding site of the receptor encompasses the phosphate group plus three of the five mannose rings of pentamannosyl phosphate. Receptor specificity for mannose arises from protein contacts with the 2-hydroxyl on the terminal mannose ring adjacent to the phosphate group. Glycosidic linkage preference originates from the minimization of unfavorable interactions between the ligand and receptor.

Recent advances in the structural analyses of both CD-MPR and CI-MPR have revealed the structural basis for phosphomannosyl recognition by these receptors and provided insights into how the receptors load and unload their cargo. A surprising finding is that the CD-MPR is dynamic, with at least two stable quaternary states, the open (ligand-bound) and closed (ligand-free) conformations, similar to those of hemoglobin. Ligand binding stabilizes the open conformation; changes in the pH of the environment at the cell surface and in endosomal compartments weaken the ligand-receptor interaction and/or weaken the electrostatic interactions at the subunit interface, resulting in the closed conformation (Kim et al. 2009).

CD-MPR Adopts at Least Two Different Conformations

Crystallographic studies of CD-MPR have identified 11 amino acids within its carbohydrate binding pocket. Mutant receptors containing a single amino acid substitution toward a lysosomal enzyme showed that substitution of Gln^{66}, Arg^{111}, Glu^{133}, or Tyr^{143} results in a >800-fold decrease in affinity, suggesting that these four amino acids are essential for carbohydrate recognition by CD-MPR. Furthermore, Asp^{103} has been identified as the key residue which mediates the effects of divalent cations on the binding properties of the CD-MPR. The MPRs encounter a variety of conditions as they travel to various compartments where they bind and release their ligands. Key to their function is pH-dependence of ligand-protein interaction. Cells treated with reagents that raise the pH of endosomal/lysosomal compartments exhibit decreased sorting of lysosomal enzymes to lysosomes and a concomitant increase in the secretion of these enzymes into the medium (Imort et al. 1983). This observation implies that it is essential for MPRs to release their ligands in the acidic environment of endosomes in order to be able to recycle back to the TGN to retrieve additional lysosomal enzymes. To determine whether different pH conditions elicit conformational changes in the receptor that alters ligand binding affinities, CD-MPR structures were obtained under different conditions representing various environments encountered by the receptor: bound state at pH 6.5 and pH 7.4 and unbound state at pH 6.5 and pH 4.8 (Olson et al. 2002, 2008) (Fig. 3.4).

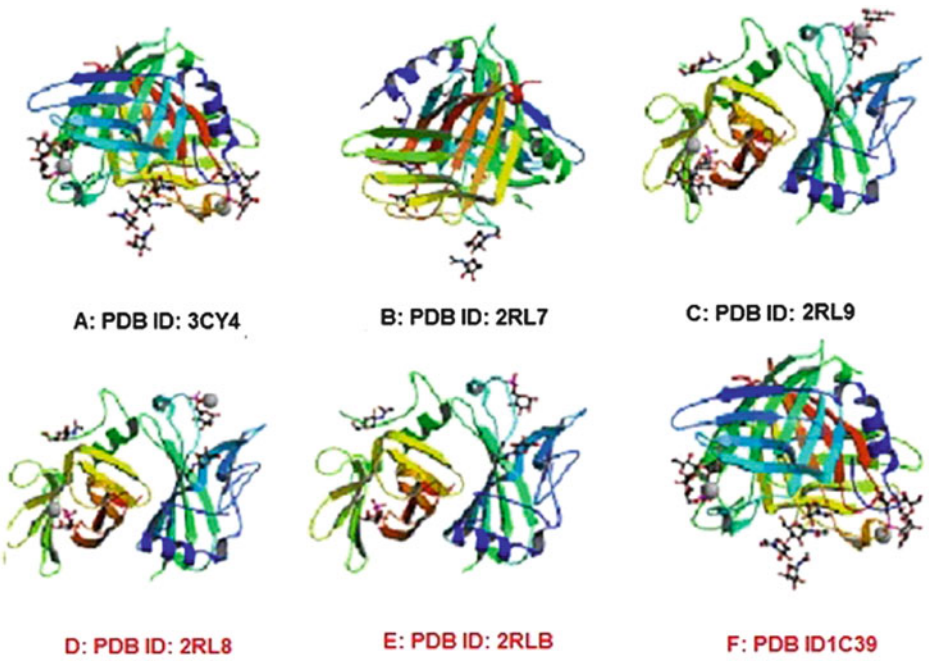

Fig. 3.4 **Crystal Structure of bovine CD-MPR.** (**a**) at pH 7.4 (Asymmetric Unit) (PDB ID: 3CY4 DOI:dx.doi.org); (**b**) at pH 4.8 (PDB ID: 2RL7); (**c**) at pH 6.5 bound to trimannoside (PDB ID: 2RL9 DOI:dx.doi.org); (**d**) at pH 6.5 bound to M6P (PDB ID: 2RL8 DOI:dx.doi.org); (**e**) at pH 6.5 bound to M6P in absence of Mn (PDB ID: 2RLB) DOI:dx.doi.org; (**f**) bound to pentamannosyl phosphate (PDB ID:1 C39)

These structures of CD-MPR were categorized into one of two conformations: an "open" conformation found in all structures containing ligand in the binding pocket and a "closed" conformation found in all structures missing bound carbohydrate (Dahms et al. 2008). Unlike what has been observed in other lectins, the structure of the ligand-free CD-MPR differs considerably from the ligand-bound form in that changes in both quaternary structure and positioning of loops involved in sugar binding, along with changes in the spacing of the two carbohydrate binding sites in the dimeric receptor (the Cα atoms of His^{105} located in Loop C are ~34 Å apart in the open conformation and ~26 Å apart in the closed conformation). Loop D (residues Glu^{134}–Cys^{141}) exhibits the most dramatic change in position, with Val^{138} displaying the largest displacement (Cα–Cα distance of 16 Å). The CD-MPR conformation differs dramatically from other lectins in an unbound state, where water molecules fill the shallow binding grooves of other most lectins in absence of bound sugar. Instead of essential side chain interactions being shifted from the carbohydrate hyroxyls to water, the pocket of CD-MPR undergoes restructuring: loop D swings into the binding pocket in the absence of ligand and provides contacts that hold essential residues in the proper orientation so that they are maintained in a "ready-state" to accept ligand. The two conformations also display a dramatic difference in their quaternary structure that can be described globally as a scissoring and twisting motion between the two subunits of the dimer. Results indicate that the CD-MPR is dynamic and must be able to transition between two conformations as it moves to different organelles, with changing environment of each (Dahms et al. 2008; Olson et al. 2008).

Based on these structures, distinct mechanisms for the dissociation of lysosomal enzymes at the cell surface and under the acidic conditions of the endosome were proposed for the CD-MPR (Olson et al. 2008). His^{105} is the only residue of the receptor in which a titratable side chain is involved in binding the phosphate group of M6P. Deprotonation of His^{105} and the phosphate moiety of M6P appear to be key elements in the release of ligand at the cell surface: loss of the electrostatic interaction between the uncharged His^{105} and M6P is predicted to facilitate dissociation of phosphorylated ligands at pH 7.4. In the acidic environment of the endosome, it is proposed that disruption, via protonation, of intermonomer electrostatic interactions that tie loop D of one monomer to the α-helix of the other monomer in the ligand bound conformation would "free" loop D to move into the binding pocket, resulting in the displacement of ligand. In addition, protonation of Glu^{133} that is located in the binding pocket is predicted to weaken its interaction with the 3- or 4-hydroxyl group of M6P and disrupt the electrostatic environment of the entire binding pocket, thereby enhancing the release of M6P. The repositioning of loop D into the binding pocket eliminates its intermonomer interaction with the N-terminal α-helix. This loss of intermonomer contact may trigger the

reorientation of the two monomers as the receptor changes its quaternary structure, adopting a more closed conformation in the unbound state (Dahms et al. 2008; Olson et al. 2008). These results allowed to suggest that the receptor regulates its ligand binding upon changes in pH; the pK_a of Glu^{133} appeared to be responsible for ligand release in the acidic environment of the late endosomal compartment, and the pK_a values of the sugar phosphate and His^{105} were accountable for its inability to bind ligand at the cell surface where the pH was about 7.4.

Sequence comparison between CD-MPR and CI-MPR shows that they are related. In fact, the extracytoplasmic domain of CD-MPR is homologous to the approximately 145 amino acid long repeating domains present in the CI-MPR with sequence identity ranging from 14% to 28%. These studies allowed to conclude that these two receptors located on different chromosomes (12p13 and 6q26, respectively) have diverged from a common ancestral gene (Dahms et al. 1987; Klier et al. 1991). This receptor is a 46 kDa single polypeptide chain that contains a putative signal sequence and a transmembrane domain. The CD-MPR is a single membrane-spanning domain, which separates a N-terminal extracytoplasmic region with five potential Asn-linked glycosylation sites, from a C-terminal cytoplasmic region without Asn-glycosylation sites. Sequence analysis of the bovine CD-MPR revealed that it consists of a 28 amino-acid residue N-terminal signal sequence, a 159 amino acid residue luminal domain, a 25 amino acid residue transmembrane domain and a 67 amino acid residue C-terminal cytoplasmic domain. It is highly conserved from mouse to human (93% homology). The CD-MPR appears to be a homodimer at the membrane (Stein et al. 1987a), and either a dimer or a tetramer in solution (Dahms et al. 1987; Tong and Kornfeld 1989; Stein et al. 1987b).

3.4.3 Carbohydrate Binding Sites in MPRs

Soluble acid hydrolases constitute a group of over 60 heterogeneous enzymes that differ in size, oligomeric state, number of *N*-linked oligosaccharides, extent of phosphorylation, and the position of the M6P moiety and its linkage to the penultimate mannose residue in the oligosaccharide chain (Dahms et al. 2008). The two MPRs have been shown to display different affinities and capacities for transport of various acid hydrolases, and both receptors are necessary for the efficient sorting of all lysosomal enzymes to the lysosome as neither MPR can fully compensate for the other (Dahms et al. 2008). These studies indicate that the two MPRs recognize distinct but overlapping populations of acid hydrolases. A proteomic analysis of serum from mutant mice deficient in either the CD-MPR or CI-MPR revealed that several lysosomal proteins are preferentially sorted by the CD-MPR (e.g., tripeptidyl peptidase I) or CI-MPR (e.g., cathepsin D) (Qian et al. 2008). Amine-activated glycans, covalently printed on N-hydroxysuccinimide-activated glass slides, interrogated with different concentrations of rCD-MPR or soluble CI-MPR. Neither receptor bound to non-phosphorylated glycans. The CD-MPR bound weakly or undetectably to the phosphodiester derivatives, but strongly to the phosphomonoester-containing glycans with the exception of a single Man7GlcNAc2-R isomer that contained a single M6P residue. By contrast, the CI-MPR bound with high affinity to glycans containing either phospho-mono- or -diesters although, like the CD-MPR, it differentially recognized isomers of phosphorylated Man7GlcNAc2-R. This differential recognition of phosphorylated glycans by the CI- and CD-MPRs has implications for understanding the biosynthesis and targeting of lysosomal hydrolases (Song et al. 2009). Future studies will shed light onto the functional significance of two distinct MPRs in a given cell type.

3.4.4 Similarities and Dis-similarities between two MPRs

The CD-MPR and CI-MPR share a number of similarities with respect to carbohydrate recognition. For example, both MPRs bind the M6P with essentially the same affinity ($7–8 \times 10^{-6}$ M). Mannose or glucose 6-phosphate interact poorly with the MPRs ($K_i = 1–5 \times 10^{-2}$ M) (Tong et al. 1989; Tong and Kornfeld 1989). Like mammalian MPRs, calotes MPR-300/IGF-IIR also binds IGF-II with high affinity (K_D ~12.02 nM). A number of synthetic analogs and those with the highest affinity to the CI-MPR were found to be isosteric to M6P. Several M6P analogues with phosphonate, carboxylate or malonate groups displayed a higher affinity and a stronger stability in human serum than M6P itself. These derivatives could be used to favour the delivery of specific therapeutic compounds to lysosomes, notably in enzyme replacement therapies of lysosomal diseases or in neoplastic drug targeting. Although analogues containing two negative charges were the best ligands, the presence of a phosphorous atom was not necessary for recognition (Gary-Bobo et al. 2007). In addition, linear mannose sequences which contain a terminal M6P linked α1, 2 to the penultimate mannose were shown to be the most potent inhibitors (Distler et al. 1991; Tomoda et al. 1991), suggesting that the MPRs bind an extended oliogosaccharide structure which includes the M6P α1,2 Man sequence. The crystal structure of CD-MPR complexed with α1, 2-linked phosphorylated trimannoside has revealed the site of penultimate and prepenultimate mannose rings in the binding pocket and their hydrogen bond interactions with the receptor (Olson et al. 2008). Furthermore, multivalent interactions

between the receptor and a lysosomal enzyme result in high affinity binding, of the order of 1–10 nM for both MPRs (Dahms et al. 2008; Watanabe et al. 1990). The two MPRs exhibit optimal ligand binding at ~pH 6.4 and no detectable binding below pH 5, which is in accordance with their function of releasing ligands in acidic environment of the endosome.

In contrast to these similarities, the two MPRs differ in their binding properties, which depend on pH, cations, and nature of phosphodiesters. The CI-MPR retains phosphomannosyl binding capabilities at neutral pH which corresponds well with the ability of this receptor to bind and internalize lysosomal enzymes at the cell surface. In contrast, the ligand binding ability of the CD-MPR is dramatically reduced at a pH $>$ 6.4 (Tong et al. 1989; Tong and Kornfeld 1989) which is consistent with its decreased ability to bind and internalize lysosomal enzymes at the cell surface (Stein et al. 1987a, b). The inability to purify the CD-MPR by phosphomannosyl affinity chromatography performed in the absence of cations led to its designation as a "cation-dependent" receptor (Hoflack and Kornfeld 1985a, b). However, the presence of cations increases the binding affinity of the CD-MPR towards M6P (Tong and Kornfeld 1989) and lysosomal enzymes only fourfold (Sun et al. 2005) but has no effect on the binding affinity of CI-MPR. This finding differentiates the CD-MPR from C-type lectins which have an absolute requirement for calcium to carry out their sugar binding activities. Mutagenesis studies (Sun et al. 2005) indicated that a conserved aspartic acid residue at position 103 of the CD-MPR, which is not present in the CI-MPR, necessitates the presence of a divalent cation in the binding pocket to obtain high affinity ligand binding by functioning to neutralize the negative charge of Asp^{103} juxtaposed to the phosphate oxygen of M6P. The CI-MPR, unlike the CD-MPR, is able to recognize MP-GlcNAc phosphodiesters as well as lysosomal enzymes derived from *Dictyostelium discoideum* which contain mannose 6-sulfate residues and small methyl phosphodiesters, M6P-OCH_3, but not phosphomonoesters (c/r Dahms et al. 2008).

3.5 Functional Mechanisms

3.5.1 Sorting of Cargo at TGN

At TGN, sorting of cargo to different destinations is regulated by several mechanisms: (1) biochemically distinct coats can specify protein sorting,; (2) cytosol-oriented sorting signals of cargo proteins direct them to the appropriate export site; (3) TGN might be organized into discrete subdomains dedicated to assemble specific coat population (Traub and Kornfeld 1997). The constitutive pathway in polarized epithelial cells (e.g. MDCK cells) includes the apical- and basolateral routes. Sorting to the basolateral pathway is mediated by cytoplasmic sorting signals of the cargo molecules. They include tyrosine residues, the dileucine motif, or "adders" that contains neither dileucine- or tyrosine-motifs (Keller and Simons 1997). The machinery responsible for the basolateral sorting is currently unknown while some data implicate that it could be mediated by AP-1 and clathrin (Futter et al. 1998). However, unequivocal evidence showing that the TGN contains sorting mechanisms able to discriminate between proteins traveling to apical and basolateral surfaces has not been obtained. Sorting signals involved in the targeting to selective pathway, among others, include the M6P residues in lysosomal hydrolases and tyrosine- and di-leucine-based sorting determinants in membrane proteins which direct them into CCVs and further into the endosomal/lysosomal system (Kirchhausen et al. 1997; Marks et al. 1997; Rohn et al. 2000). The membrane proteins are e.g. the lysosomal associated membrane protein (LAMP) and the lysosomal integral membrane protein (LIMP). A conformation-dependent motif is suggested to destine the proteins for secretion for a regulated secretory pathway by Keller and Simons (1997).

At TGN, the sorting and transport of a group of soluble proteins via a selective pathway to lysosomes relies on the existence of M6P residues on their oligosaccharides (Le Borgne and Hoflack 1998a, b). They serve as recognition signals for MPRs. Sorting of MPRs and their bound ligands to their destinations is mediated preferentially by the interaction of tyrosine- and dileucine-based sorting signals present in their tails with the adaptor protein complex AP-1 and by transport in CCVs (Le Borgne et al.1996; Le Borgne and Hoflack 1998a). The pinching off of the CCVs from the membranes is effected by dynamin (Jones et al. 1998; Kasai et al. 1999).

Though the CD-MPR and the CI-MPR deliver soluble acid hydrolases to lysosome in higher eukaryotic cells by binding with high affinity to M6P residues, found on N-linked oligosaccharides of their ligands, for many other transmembrane proteins, the MPRs contain multiple molecular sorting signals in their cytoplasmic domains that mediate their intracellular traffic between distinct membrane-bound compartments (Lobel et al. 1989). A schematic representation of the different vesicle transport pathways originating from TGN is given (Fig. 3.1).

3.5.1.1 Interaction of Phosphorylated Oligosaccharides and Lysosomal Enzymes with CD-MPR and CI-MPR

Oligosaccharides with phosphomonoesters interact with the CD-MPR, and molecules with two phosphomonoesters showed the best binding. Lysosomal enzymes with several oligosaccharides containing only one phosphomonoester had a higher affinity for the receptor than did the isolated

oligosaccharides, indicating the possible importance of multivalent interactions between weakly binding ligands and the receptor. The binding of phosphorylated lysosomal enzymes to the CD-MPR is markedly influenced by pH. At pH 6.3, almost all of the lysosomal enzymes bound to the receptor. Results indicated that at neutral pH the phosphorylated oligosaccharides on some lysosomal enzyme molecules are oriented in a manner which makes them inaccessible to the binding site of the CD-MPR. Since the same enzymes bind to the CI-MPR at neutral pH, at least a portion of the phosphomannosyl residues must be exposed. It appeared that small variations in the pH of the Golgi compartment where lysosomal enzymes bind to the receptors could potentially modulate the extent of binding to the two receptors (Hoflack et al. 1987).

Lysosomal enzymes bearing phosphomannosyl residues bind specifically to MPRs in the Golgi apparatus and the resulting receptor-ligand complex is transported to an acidic prelyosomal compartment where the low pH mediates the dissociation of the complex (Le Borgne and Hoflack 1998). The transport of proteins from the secretory to the endocytic pathway is mediated by carrier vesicles coated with the AP-1 Golgi assembly proteins and clathrin. The MPRs are segregated into these transport vesicles. Together with GTPase-ARF-1, these cargo proteins are essential components for the efficient translocation of cytosolic AP-1 onto membranes of the TGN, the first step of clathrin coat assembly. The transport of lysosomal enzymes to lysosomes requires two distinct determinants in the CD-MPR carboxyl-terminal domain, a casein kinase II phosphorylation site critical for the efficient interaction of AP-1 with its target membranes and the adjacent di-leucine motif which appears more important for a post AP-1 binding step in the CD-MPR cycling pathway.

3.5.2 TGN Exit Signal Uncovering Enzyme

According to Ghosh et al. (2003) dynamic fusion/fission between the late endosomal and lysosomal compartments results in selective delivery of the hydrolases to the lysosome. TIP47/Rab9 prevents the MPRs from reaching the lysosomes, in which they would otherwise be degraded. The return pathway from the early endosomal compartment to the Golgi is probably mediated by PACS-1-assisted packaging into AP1-containing CCVs, whereas that from the late endosomal (LE) compartments is mediated by TIP47 and Rab9. Some of the MPRs go to the cell surface either from early or late endosomes through the recycling endosome (RE), or from proximal TGN cisternae as a consequence of mis-sorting. The cell-surface receptors are internalized in AP2 CCVs and delivered back to the endosomes.

Nair et al. (2005) proposed that the human M6P uncovering enzyme participates in the uncovering of M6P recognition tag on lysosomal enzymes, a process that facilitates recognition of those enzymes by MPRs to ensure a delivery to lysosomes. Uncovering enzyme has been identified on TGN. The cytoplasmic tail of the uncovering enzyme does not possess any of the known canonical signal sequences for interaction with Golgi-associated γ-ear-containing adaptor proteins. The identification of a TGN exit signal in its cytoplasmic tail elucidates the trafficking pathway of uncovering enzyme, a crucial player in the process of lysosomal biogenesis (Nair et al. 2005). However, uncovered phosphates are not essential for optimal recognition by the phosphomannosyl receptor.

3.5.3 Association of Clathrin-Coated Vesicles with Adaptor Proteins

It is suggested that 46-kDa MPR contains multiple binding sites for clathrin adaptors (Honing et al. 1997). The Golgi-derived and plasma membrane-derived clathrin-coated vesicles can be distinguished by the nature of their underlying assembly proteins AP-1 and AP-2, two related heterotetrameric complexes (Robinson 1994). Localization studies are consistent with the notion that AP-1 is associated with TGN-derived vesicles, whereas AP-2 is found in plasma membrane-derived vesicles. In vitro studies have shown that the translocation of cytosolic AP-1 onto membranes requires ADP-ribosylation factor ARF-1 (Traub et al. 1993), a small GTPase also involved in coatomer binding and vesicular transport in early secretory pathway (Rothman 1994; Boman and Kahn 1995). The AP-1 complex interacts with sorting signals in the cytoplasmic tails of cargo molecules and targeted disruption of the mouse μ1A-adaptin gene causes embryonic lethality. Under normal conditions, MPRs are cargo molecules that exit the TGN via AP-1-clathrin-coated vesicles. But the steady-state distribution of MPR46 and MPR300 in μ1A-deficient cells is shifted to endosomes at the expense of TGN. Thus, MPR46 fails to recycle back from the endosome to the TGN, indicating that AP-1 is required for retrograde endosome to TGN transport of the receptor (Meyer et al. 2000).

Binding Sites in CI-MPR for AP-1

The trafficking of CI-MPR between the TGN and endosomes requires binding of sorting determinants in the cytoplasmic tail of the receptor to adaptor protein complex-1 (AP-1). A GST pull-down binding assay identified four binding motifs in the cytoplasmic tail of CI-MPR: a tyrosine-based motif ^{26}YSKV^{29}, an internal dileucine-based motif ^{39}ETEWLM^{44}, and two casein kinase 2 sites ^{84}DSEDE^{88} and ^{154}DDSDED^{159}. The YSKV motif mediated the strongest interaction with AP-1 and the two CK2 motifs bound AP-1 only when they were

phosphorylated. The COOH-terminal dileucines were not required for interaction with AP-1 (Ghosh and Kornfeld 2004).

AP-3 Adaptor Complex Defines a Novel Endosomal Exit Site

The AP-3 adaptor complex has been implicated in the transport of lysosomal membrane proteins. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins (Le Borgne et al. 1998b). Electron microscopy showed that AP-3 is associated with budding profiles evolving from a tubular endosomal compartment that also exhibits budding profiles positive for AP-1. AP-3 colocalizes with clathrin, but to a lesser extent than does AP-1. The AP-3- and AP-1-bearing tubular compartments contain low amounts of the CI-MPR and the lysosome-associated membrane proteins (LAMPs) 1 and 2. AP-3 defines a novel pathway by which lysosomal membrane proteins are transported from tubular sorting endosomes to lysosomes. In an attempt to find the site of action of AP-3 Chapuy et al. (2008) showed that sorting of TRP-1 and CD-MPR was AP-1 dependent, while budding of tyrosinase and LAMP-1 required AP-3. Depletion of clathrin inhibited sorting of all four cargo proteins, suggesting that AP-1 and AP-3 are involved in the formation of distinct types of CCVs, each of which is characterized by the incorporation of specific cargo membrane proteins. Harasaki et al. (2005) indicated that three proteins: CI-MPR, carboxypeptidase D (CPD) and low-density lipoprotein receptor-related protein 1 (LRP1) have AP-dependent sorting signals, which may help to explain the relative abundance of AP complexes in CCVs.

AP-4 as a Component of the Clathrin Coat Machinery

AP-4, a protein complex related to clathrin adaptors (Dell'Angelica et al. 1999) is localized mainly in the Golgi complex, as well as on endosomes and transport vesicles. Interestingly, AP-4 is localized with the clathrin coat machinery in the Golgi complex and in the endocytic pathway. Moreover, AP-4 is localized with the CI-MPR, but not with the transferrin receptor, LAMP-2 or invariant chain. The difference in morphology between CI-MPR/AP-4-positive vesicles and CI-MPR/AP-1-positive vesicles raises the possibility that AP-4 acts at a location different from that of AP-1 in the intracellular trafficking pathway of CI-MPR (Barois and Bakke 2005).

3.5.4 Role of Di-leucine-based Motifs in Cytoplasmic Domains

Ludwig et al. (1993) indicated that CD-MPR is required for efficient intracellular targeting of multiple lysosomal enzymes, although homozygous mice lacking CD-MPR suggested that other targeting mechanisms could partially compensate for the loss of CD-MPR in vivo. The cytoplasmic domain of the M6P/IGF2R has two signals for lysosomal enzyme sorting in the Golgi, a di-leucine-based motif (LLHV sequence) and the tyrosine-based endocytosis motif (YKYSKV sequence) (Johnson and Kornfeld 1992), whereas a di-leucine-based motif near the carboxyl terminus of the CD-MPR (HLLPM sequence) in cytoplasmic domain is essential for efficient targeting of newly synthesized lysosomal enzymes (Johnson and Kornfeld 1992). Several other transmembrane proteins destined to the lysosomes also contain di-leucine-based motifs in their cytoplasmic domains that are essential for their proper delivery to lysosomes (Sandoval and Bakke 1994). In the light of these different results, it has been proposed that di-leucine-based motifs mediate sorting of membrane proteins in the TGN. In both MPRs, the di-leucine motifs are flanked by casein kinase II phosphorylation sites that are phosphorylated in vivo (Méresse et al. 1990; Hemer et al. 1993). Such a post-translational modification occurs when the M6P/IGF2R exits from the TGN and represents a major, albeit transient, modification (Méresse and Hoflack 1993). Thus far, the functional importance of the phosphorylation sites in the M6P/IGF2R trafficking has remained controversial (Johnson and Kornfeld 1992; Chen et al. 1993). Mouse L cells deficient in the M6P/IGF-IIR were transfected with normal bovine CD-MPR cDNA or cDNAs containing mutations in the 67-amino acid cytoplasmic tail and assayed for their ability to target the lysosomal enzyme cathepsin D to lysosomes. Mutant receptors with the carboxyl-terminal His-Leu-Leu-Pro-Met67 residues deleted or replaced with alanines sorted cathepsin D below the base-line value (Johnson and Kornfeld 1992). Of the eight amino acids mutated in bovine CD-MPR, four (Gln^{66}, Arg^{111}, Glu^{133}, and Tyr^{143}) were found to be essential for ligand binding. In addition, mutation of the single histidine residue, His^{105}, within the binding site diminished the binding of the receptor to ligand, but did not eliminate the ability of the CD-MPR to release ligand under acidic conditions (Olson et al. 1999a).

A casein kinase II (CK-II) phosphorylation site in the cytoplasmic tail of CD-MPR determines the interaction of AP-1 Golgi assembly proteins with membranes. Mauxion et al. (1996) demonstrated that the casein kinase II phosphorylation site in the CD-MPR cytoplasmic domain determines the high affinity of AP-1 for membranes and that mutations introduced independently in the tyrosine-based or the di-leucine-based motifs are not sufficient to modify these interactions. MPR-negative fibroblasts have a low capacity of recruiting AP-1 which can be restored by re-expressing the MPRs in these cells. This property helped to identify the protein motif of the CD-MPR

cytoplasmic domain that is essential for these interactions. It was found that the targeting of lysosomal enzymes requires the CD-PDR cytoplasmic domain that is different from tyrosine-based endocytosis motifs. The first is a casein kinase II phosphorylation site (ESEER) probably acts as a dominant determinant controlling CD-MPR sorting in the TGN. The second is the adjacent di-leucine motif (HLLPM), which, by itself, is not critical for AP-1 binding, but is absolutely required for a downstream sorting event (Mauxion et al. 1996).

Domain 5 of CD-MPR Preferentially Binds Phosphodiesters: Sequence alignment predicts that domain 5 contains four conserved residues (Gln, Arg, Glu, Tyr) which are essential for M6P binding by the CD-MPR and domains 1–3 and 9 of the CI-MPR. Surface plasmon resonance (SPR) analyses of constructs containing single amino acid substitutions showed that these conserved residues (Gln^{644}, Arg^{687}, Glu^{709}, Tyr^{714}) are critical for carbohydrate recognition by domain 5. Furthermore, the N-glycosylation site at position 711 of domain 5, which is predicted to be located near the binding pocket, has no influence on the carbohydrate binding affinity. Using endogenous ligands for the MPRs demonstrated that, unlike the CD-MPR or domain 9 of the CI-MPR, domain 5 exhibits a 14–18-fold higher affinity for MP-GlcNAc than M6P, implicating this region of the receptor in targeting phosphodiester-containing lysosomal enzymes to the lysosome (Chavez et al. 2007). Crystallographic studies have shown that at pH 6.5, the CD-MPR bound to M6P adopts a significantly different quaternary conformation than the CD-MPR in a ligand-unbound state, a feature unique among known lectin structures. Additional crystal structures of the available CD-MPR revealed the positional invariability of specific binding pocket residues which implicate intermonomer contact(s), as well as the protonation state of M6P, as regulators of pH-dependent carbohydrate binding (Olson et al. 2008).

Interaction of MPRs with GGA Proteins: The GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding), the multidomain family of proteins have been implicated in protein trafficking between Golgi and endosomes. Evidence suggests that CI-MPR and CD-MPR bind specifically to VHS domains of GGAs through acidic cluster-dileucine motifs at the carboxyl ends of their cytoplasmic tails. However, the CD-MPR binds VHS domains more weakly than the CI-MPR. Alignment of C-terminal residues of two receptors revealed a number of non-conservative differences in the acidic cluster-dileucine motifs and the flanking residues. Studies indicate that GGAs participate in lysosomal enzyme sorting mediated by CD-MPR (Doray et al. 2002).

3.5.5 Sorting Signals in Endosomes

The endocytosis of cell surface proteins is mediated by tyrosine-based (Trowbridge et al. 1993) or di-leucine-based motifs (Sandoval and Bakke 1994). In case of CI-MPR/IGF2 receptor, its endocytosis requires a single YSKV sequence (Jadot et al. 1992), while that of CD-MPR requires two distinct motifs. The bovine CD-MPR cycles between TGN, endosomes and the plasma membrane. When the terminal 40 residues were deleted from the 67-amino acid cytoplasmic tail of the CD-MPR, the half-life of the receptor was drastically decreased and the mutant receptor was recovered in lysosomes; amino acids 34–39 being critical for avoidance of lysosomal degradation. Findings indicated that the cytoplasmic tail of the CD-MPR contains a signal that prevents the receptor from trafficking to lysosomes. The transmembrane domain of the CD-MPR also contributes to this function (Rohrer et al. 1995).

The 67-amino acid cytoplasmic tail of CD-MPR contains a signal(s) that prevents the receptor from entering lysosomes where it would be degraded. A receptor with a Trp^{19} –> Ala substitution in the cytoplasmic tail resulted into highly missorted to lysosomes whereas receptors with either Phe^{18} –> Ala or Phe^{13} –> Ala mutations were partially defective in avoiding transport to lysosomes. Results indicated that the di-aromatic motif (Phe^{18}-Trp^{19} with Trp^{19} as the key residue) in its cytoplasmic tail is required for the sorting of the receptor from late endosomes back to the Golgi apparatus. Because a diaromatic amino acid sequence is also present in the cytoplasmic tail of other receptors known to be internalized from the plasma membrane, this feature may be a general determinant for endosomal sorting (Schweizer et al. 1997). However, the CI-MPR lacks such a di-aromatic motif. Studies indicate that sorting of the CD-MPR in late endosomes requires a distinct di-aromatic motif with only limited possibilities for variations, in contrast to the CI-MPR, which seems to require a putative loop (Pro^{49}-Pro-Ala-Pro-Arg-Pro-Gly^{55}) along with additional hydrophobic residues in the cytoplasmic tail. This raises the possibility of two separate binding sites on Tip47 because both receptors require binding to Tip47 for endosomal sorting (Nair et al. 2003).

3.5.6 Palmitoylation of CD-MPR is Required for Correct Trafficking

Evasion of lysosomal degradation of the CD-MPR requires reversible palmitoylation of a cysteine residue in its cytoplasmic tail. Because palmitoylation is reversible and essential for correct trafficking, it presents a potential regulatory mechanism for the sorting signals within the cytoplasmic domain of

the CD-MPR. The two cysteine residues (Cys^{30} and Cys^{34}) in the cytoplasmic tail of the CD-MPR are palmitoylated via thioesters and Cys^{34} residue influences the biologic function of the receptor. Mutation of Cys^{34} to Ala resulted in the gradual accumulation of the receptor in dense lysosomes and the total loss of cathepsin D sorting function in the Golgi. A Cys^{30} to Ala mutation had no biologic consequences, showing the importance of Cys^{34}. Mutation of amino acids 35–39 to alanines impaired palmitoylation of Cys^{30} and Cys^{34} and resulted in abnormal receptor trafficking to lysosomes and loss of cathepsin D sorting. The palmitoylation of Cys^{30} and Cys^{34} leads to anchoring of this region of the cytoplasmic tail to the lipid bilayer. Thus, anchoring via Cys^{34} is essential for the normal trafficking and lysosomal enzyme sorting function of the receptor (Schweizer et al. 1996). The palmitoylation of the CD-MPR occurs enzymatically by a membrane-bound palmitoyltransferase, which cycles between endosomes and the plasma membrane. The localization of the palmitoyltransferase indicates it as a regulator of the intracellular trafficking of the CD-MPR and also affects the sorting/activity of other receptors cycling through endosomes (Stöckli and Rohrer 2004).

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