

Phosphorylation of the Cation-independent Mannose 6-Phosphate Receptor Is Closely Associated with Its Exit from the *trans*-Golgi Network

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Abstract. We have previously shown that two serine residues present in two conserved regions of the bovine cation-independent mannose 6-phosphate receptor (CI-MPR) cytoplasmic domain are phosphorylated *in vivo* (residues 2421 and 2492 of the full length bovine CI-MPR precursor). In this study, we have used CHO cells to investigate the phosphorylation state of these two serines along the different steps of the CI-MPR exocytic and endocytic recycling pathways. Transport and phosphorylation of the CI-MPR in the biosynthetic pathway were examined using deoxymannojirimycin (dMM), a specific inhibitor of the *cis*-Golgi processing enzyme α -mannosidase I which leads to the accumulation of N-linked high mannose oligosaccharides on glycoproteins. Upon removal of dMM, normal processing to complex-type oligosaccharides (galactosylation and then sialylation) occurs on the newly synthesized glycoproteins, including the CI-MPR which could then be purified and analyzed on lectin affinity columns. Phosphorylation of the newly

synthesized CI-MPR was concomitant with the sialylation of its oligosaccharides and appeared as a major albeit transient modification. Phosphorylation of the cell surface CI-MPR was examined during its endocytosis as well as its return to the Golgi using antibody tagging and exogalactosylation. The cell surface CI-MPR was not phosphorylated when it entered clathrin-coated pits or when it moved to the early and late endosomes. In contrast, the surface CI-MPR was phosphorylated when it had been resialylated upon its return to the *trans*-Golgi network. Subcellular fractionation experiments showed that the phosphorylated CI-MPR and the corresponding kinase were found in clathrin-coated vesicles. Collectively, these results indicate that phosphorylation of the two serines in the CI-MPR cytoplasmic domain is associated with a single step of transport of its recycling pathways and occurs when this receptor is in the *trans*-Golgi network and/or has left this compartment via clathrin-coated vesicles.

THE mannose 6-phosphate receptors (MPRs)¹ are essential components that allow lysosomal enzymes to reach the lysosomes (for review see Kornfeld 1992; Kornfeld and Mellman, 1989; von Figura, 1991). Their main function in this targeting process is to divert the soluble phosphorylated lysosomal enzymes from the bulk flow secretory pathway. This occurs in the *trans*-Golgi network (TGN) where the MPRs and their ligands are sorted into clathrin-coated vesicles which bud and then fuse with endosomes. The MPRs release their ligands in these acidified compartments, and return to the TGN to repeat several rounds of this process. Two MPRs that recognize mannose 6-phosphate residues have been described (for review see also Dahms et al., 1989). The first, the cation-independent mannose 6-phosphate receptor (CI-MPR) is a large mole-

cule of \approx 300 kD. The CI-MPR has a major role in intracellular retention of the newly synthesized lysosomal enzymes (Kyle et al., 1988; Lobel et al., 1989). In addition, this receptor can also bind the insulin-like growth factor II at the cell surface; however, the biological significance of this dual function remains to be elucidated. The second receptor, the cation-dependent mannose 6-phosphate receptor (CD-MPR) is a smaller molecule made of at least two identical subunits of 46 kD. Its role in lysosomal transport is not as firmly established but overexpression experiments suggest that this receptor is not only involved in intracellular retention of newly synthesized lysosomal enzymes (Watanabe et al., 1990) but also in their secretion (Chao et al., 1990). The two MPRs are also present on the cell surface where they are internalized via clathrin-coated pits before their delivery into early endosomes. The endocytosed MPRs either recycle back to the plasma membrane, like other receptors specialized in endocytosis, or return to the TGN to participate in sorting of the newly synthesized lysosomal enzymes (Duncan and Kornfeld, 1988).

1. *Abbreviations used in this paper:* CI-MPR, cation-independent MPR; CD-MPR, cation-dependent MPR; dMM, deoxymannojirimycin; LFA, *Lizobryon flavus* agglutinin; MPR, mannose 6-phosphate receptor; RCA-I, *Ricinus communis* agglutinin; TGN, *trans*-Golgi network.

To carry out their function and to be properly routed from one compartment to another, the MPRs must use different determinants in their cytoplasmic domains that are recognized by specific cytosolic components. Thus, it has become clear during the last years that rapid endocytosis of the CI-MPR (Lobel et al., 1989; Canfield et al., 1991; Jadot et al., 1992), like that of other plasma membrane receptors, is triggered by a short protein motif containing a tyrosine residue and shaped in a tight turn conformation (Bansal and Gierasch, 1991; Eberle et al., 1991). Efficient endocytosis of the CD-MPR appears to require two independent signals, one containing phenylalanine residues and the other a tyrosine (Johnson et al., 1990). Pearse (1988) has shown that the endocytosis signal present in the cytoplasmic domains of receptors recycling at the plasma membrane are recognized by the plasma membrane-specific adaptors (also called HA II or AP II) which together with clathrin form the coat of the plasma membrane-derived vesicles (for review see Pearse and Robinson, 1990). Therefore, it appears that these tyrosine-dependent interactions mediate the recruitment of plasma membrane receptors into endocytic vesicles. The molecular events involved in the other steps of the MPR recycling pathways (departure from the TGN and/or recycling from endosomes) are not as well defined. It appears that the carboxyl terminal domain of the CI-MPR is crucial for efficient delivery of lysosomal enzymes to lysosomes (Lobel et al., 1989) but the precise nature of the signals involved in this complex process remains to be established. Pearse and colleagues have also proposed that sorting of the CI-MPR in the TGN and its clustering into Golgi-derived clathrin-coated vesicles are mediated by interactions of a tyrosine-independent signal present in its cytoplasmic domain with the Golgi-specific adaptors HA I or AP I (Glickman et al., 1989).

We have previously shown that the bovine CI-MPR is phosphorylated *in vivo* on two serines in its cytoplasmic domain (residues 2421 and 2492 of the full-length CI-MPR precursor according to Lobel et al., 1988) (Méresse et al., 1990). These serines are located within two regions which are highly conserved between bovine (Lobel et al., 1988), human (Oshima et al., 1988), rat (Mac Donald et al., 1988), and mouse (Ludwig, T., and P. Lobel, unpublished results) CI-MPRs. This carboxyl terminal domain was shown to be crucial for efficient intracellular retention of lysosomal enzymes (Lobel et al., 1989). We have also shown that a casein kinase II-type enzyme, highly enriched in clathrin-coated vesicles and tightly associated with the Golgi-specific adaptors, is able to phosphorylate these serines *in vitro*. These observations suggest that, *in vivo*, the CI-MPR is phosphorylated on these sites at the exit of the *trans*-Golgi network. We have investigated the phosphorylation of these serine residues during the CI-MPR recycling pathways *in vivo*. We now report that phosphorylation of the CI-MPR on these sites is a major and transient modification, closely associated with its departure from the TGN and/or its transport to endosomes via clathrin-coated vesicles. In contrast, this phosphorylation does not occur during endocytosis of the CI-MPR or when it travels through the early and late endosomes. We speculate that phosphorylation of the CI-MPR tail may modulate its interactions with the sorting machinery and/or generate an additional signal required for its next step of transport.

Materials and Methods

Materials

Limax flavus agglutinin (LFA) was purchased from Calbiochem (Bad Soden, Germany), deoxymannojirimycin (dMM) from Boehringer Mannheim GmbH (Mannheim, Germany) and sialic acid from Fluka (Neu-Ulm, Germany). Asialofetuin, galactosyltransferase, *Ricinus communis* agarose (RCA-I), UDP-galactose and D₂O were from Sigma (Deisenhofen, Germany). Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden) and Affigel from Bio-Rad (München, Germany). Secondary antibodies were from Jackson Immuno Research Laboratories, Inc. (Dianova, Hamburg, Germany). [³⁵S]methionine, [³²P]orthophosphate, and UDP-[6-³H]galactose were purchased from Amersham Buchler GmbH (Braunschweig, Germany). CMP-[4,5,6,7,8,9-¹⁴C]sialic acid was obtained from New England Nuclear (Dreieich, Germany). Soluble fragments corresponding to the extracytoplasmic domain of the CI-MPR were purified by affinity chromatography on phosphomannan-Sepharose column (Ludwig et al., 1991). The phosphomannans from *Hansenula holstii* were kindly provided by Dr. Slodki (Peoria, IL).

Cells

CHO and the two corresponding galactose-deficient cells lines, ldl-D (Kingsley et al., 1986) and Lec8 (Stanley, 1981), were kindly provided by Drs M. Krieger and P. Stanley through the American Type Culture Collection (Rockville, Maryland). They were grown as a monolayer in α MEM containing antibiotics and 10% FCS. Both mutants exhibit a drastic reduction in galactosylation and sialylation of proteins and lipids but have two different underlying defects. Lec8 cells are unable to translocate UDP-galactose from the cytosol into the Golgi cisternae (Deutscher and Hirschberg, 1986). ldl-D cells cannot synthesize UDP-Gal and UDP-GalNAc due to a deficiency of the UDP-Gal/UDP-GalNAc 4-epimerase which converts UDP-Glc and UDP-GlcNAc into UDP-Gal and UDP-GalNAc. Since this defect can be bypassed by the degradation of serum glycoproteins this phenotype is only observed in ldl-D cells grown in low concentration of serum. Normal glycosylation of N-linked oligosaccharides can be restored in ldl-D cells by addition of galactose to the culture medium. MDBK cells were grown in DME containing antibiotics and 10% FCS.

Metabolic Labeling

CHO and Lec8 cells at ~80% confluency were metabolically labeled overnight with either [³⁵S]methionine at 0.1 mCi/ml in 20% α MEM-80% α MEM lacking methionine, 5% dialyzed FCS or 0.2 mCi/ml [³²P]orthophosphate in 20% α MEM-80% phosphate-free MEM, 5% dialyzed FCS. ldl-D cells were seeded at high density in 35-mm dishes and grown for 3 d in α MEM containing 1% dialyzed FCS, antibiotics, and 1 mM dMM. For [³⁵S]methionine labeling, cells were incubated for 20 min in α MEM lacking methionine and then pulsed for 1 h in the same medium containing 0.1 mCi/ml [³⁵S]methionine, 5% dialyzed FCS, and 15 μ M galactose. After washing twice with PBS, the chase was started by adding α MEM, 5% FCS, and 15 μ M galactose. For phosphorylation experiments, cells were prelabeled for 2 h in 1 ml phosphate-free MEM, 1% dialyzed FCS, 1 mM dMM, and 0.2 mCi/ml [³²P]orthophosphate. The pulse was initiated by washing dishes twice with phosphate-free MEM and adding 1 ml phosphate-free MEM, 5% dialyzed FCS, 0.2 mCi/ml [³²P]orthophosphate, and 15 μ M galactose. When indicated, protein synthesis was inhibited after 1 h of labeling by adding 10 μ g/ml cycloheximide. In controls, cells were pulsed-chased in the same medium without galactose and containing 1 mM dMM.

Exogalactosylation

Exogalactosylation of Lec8 cells was carried out essentially as described (Brändli et al., 1990). Labeled cells grown in 35-mm dishes were washed twice with ice-cold E buffer (10 mM Hepes, pH 7.3, 150 mM NaCl, 0.1% [wt/vol] BSA). 0.8 ml of E buffer supplemented with 10 mM MnCl₂, 0.25 U/ml galactosyltransferase, and 1 mM UDP-Gal were added and dishes incubated for 1 h at 4°C. The reaction was stopped by washing the dishes twice with E buffer. [³⁵S]methionine and ³²P-labeled cells were returned to 37°C by adding pre-warmed culture medium or prewarmed labeling medium respectively and placed in the cell culture incubator for the indicated time. CI-MPR was then analyzed for galactosylation and sialylation. In controls, galactosyltransferase was omitted.

Lectin Analysis

After the indicated time, cells were washed twice with cold PBS and lysed in 1 ml of buffer 1 (50 mM Tris/HCl, pH 7.3, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10 mM β -glycerophosphate and NaF, 1 mM PMSF and Benzamide). After 1 h on ice, the insoluble material was removed by centrifugation at 13,000 rpm in a table top centrifuge. CI-MPR was isolated from cell extracts by affinity chromatography on phosphomannan-Sepharose as described (Hoflack and Kornfeld, 1985). Beads (0.1-ml bed volume) were washed six times with buffer 1, once with buffer 2 (buffer 1 with 0.1% Triton X-100), and CI-MPR was eluted by $2 \times 150 \mu\text{l}$ of buffer 2 containing 5 mM mannose 6-phosphate. Sialylated receptors were isolated by chromatography on LFA-affigel as described by Goda and Pfeffer (1988). CI-MPR was incubated for 2 h at room temperature in the presence of $50 \mu\text{l}$ LFA-affigel 1:1 slurry. Lectin matrix was washed three times with buffer 1, once with buffer 2, and eluted with 0.4 ml of buffer 2 containing 20 mM sialic acid. Unbound CI-MPR was then incubated with $50 \mu\text{l}$ of RCA-I-agarose slurry for 1 h at room temperature. Beads were washed as described for the LFA-affigel and galactosylated receptor was eluted with 0.4 ml of buffer 2 containing 0.1 M galactose. LFA eluted, RCA-I eluted and unbound CI-MPR were TCA precipitated and analyzed on 6.5% SDS-PAGE. Gels were dried and autoradiographed.

Antibody Tagging of the Cell Surface CI-MPR

Labeled CHO cells in 35-mm dishes were washed twice with ice-cold TBS (25 mM Tris/HCl, pH 7.3, 150 mM NaCl, 2.5 mM MgCl_2 and CaCl_2) and incubated 45 min on ice with 0.8 ml TBS containing 50 $\mu\text{g/ml}$ BSA and 0.25 $\mu\text{g/ml}$ immunopurified rabbit anti-CI-MPR. Cells were washed three times with TBS and incubated for 30 min on ice with 0.8 ml TBS containing 50 $\mu\text{g/ml}$ BSA and 0.2 $\mu\text{g/ml}$ CI-MPR soluble fragments. [^{35}S]methionine and ^{32}P -labeled cells were washed twice with TBS and returned at 37°C by adding prewarmed culture medium or prewarmed labeling medium, respectively, and placed in the cell culture incubator for the indicated time. Cell extracts were prepared as above described and cell surface/internalized tagged CI-MPR was immunoprecipitated by a 2 h incubation with $50 \mu\text{l}$ of protein A-Sepharose slurry. Sepharose beads were spun down and untagged CI-MPR was then immunoprecipitated from the supernatant with 0.5 μg immunopurified rabbit anti CI-MPR and $50 \mu\text{l}$ protein A-Sepharose slurry. Protein A-Sepharose beads were washed six times with buffer 1, once with buffer 2 and immunoprecipitated material was analyzed by 6.5% SDS-PAGE and autoradiography.

Stoichiometry of Phosphorylation

Experiments were essentially performed as described (Meisenhelder et al., 1989). To determine the stoichiometry of phosphorylation of the total intracellular CI-MPR (steady state), ldl-D CHO cells were labeled for 24 h with [^{32}P]orthophosphate (0.2 mM) as described above. The CI-MPR was then immunoprecipitated and subjected to SDS-PAGE together with samples containing known amounts of purified CI-MPR. The gel was stained with Coomassie blue and the amount of immunoprecipitated CI-MPR (≈ 1 –2 $\mu\text{g}/10$ -cm dish) was estimated by comparison with the standards. The specific radioactivity of the intracellular phosphate was assumed to be identical of that of the medium. Gel slices of the CI-MPR were excised and the radioactivity counted.

To determine the phosphorylation state of the newly synthesized sialylated CI-MPR, ldl-D cells were pre-labeled as follows. Cells were seeded at high density and grown for 3 d in α MEM containing 1% dialyzed FCS, antibiotics, and 1 mM dMM. The prelabeling was for 24 h in 20% α MEM–80% α MEM lacking methionine or phosphate, 1% dialyzed FCS, 1 mM dMM, and containing either 0.1 mCi/ml [^{35}S]methionine or 0.1 mCi/ml [^{32}P]orthophosphate (0.2 mM phosphate; specific radioactivity, 555 cpm/pmole). After washing twice with TBS, labeling was carried out for 3 h in the same medium without dMM but with 15 μM galactose. Cycloheximide (10 $\mu\text{g/ml}$) was added 1 h after the addition of galactose. The CI-MPR was then affinity purified on phosphomannan and the sialylated receptor was further separated on LFA-Affigel. Samples were electrophoresed on SDS-PAGE. Radioactivity of [^{32}P]labeled CI-MPR was counted. The amount of the non-sialylated CI-MPR was estimated as described above using known amounts of the purified CI-MPR as standards and the amount of newly synthesized sialylated CI-MPR was determined by densitometric scanning of the autoradiogram of the non-sialylated and sialylated [^{35}S]methionine-labeled CI-MPRs.

Subcellular Fractionation Procedures

Isolation of clathrin-coated vesicles was based on the method developed by Woodman and Warren (1991). Labeled CHO cells were washed twice with PBS, once in vesicle buffer (25 mM Mes, pH 6.5, 140 mM sucrose, 1 mM EGTA, 0.5 mM MgCl_2 , 10 mM β -glycerophosphate and NaF) and scraped in this buffer using a rubber policeman. Cells were pelleted by centrifugation for 5 min at 4°C at 3,000 rpm in a Heraeus minifuge GL. After addition of 1-ml vesicle buffer containing 1 mM PMSF and benzamide, cells were homogenized by five to ten passages through a 22G1 $\frac{1}{2}$ needle. The homogenate was centrifuged for 10 min at 4°C at 3,000 rpm. The PNS was then loaded on a 10 ml continuous gradient of 2% (wt/vol) Ficoll/9% (wt/vol) D_2O –20% Ficoll/90% D_2O in vesicle buffer containing 1 mM DTT, PMSF, and benzamide. The gradient was centrifuged in a SW-40 rotor for 16 h at 23,000 rpm, 4°C, and 1-ml fractions were collected from the top.

Miscellaneous Procedures

Immunofluorescence were performed on CHO grown on coverslips. The cell surface receptor was tagged with rabbit anti-CI-MPR antibodies and internalized as described above. Cells were then washed with ice cold PBS, fixed for 15 min in 3% paraformaldehyde in PBS, extensively washed with 0.1 M glycine in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Cells were incubated for 1 h with a mouse anti CI-MPR, extensively washed with PBS and incubated 1 h with secondary antibodies. Coverslips were washed with PBS and mounted in Moviol. Pictures were taken with TMY-400 films.

The CI-MPR kinase activity was determined as previously described (Mérèse et al., 1990) using a peptide corresponding to the 20 COOH-terminal amino acid residues of the bovine CI-MPR as substrate (AAATPISTFHDDSDLEDLLHV). In brief, 2 μl of fraction were added to 18 μl of kinase buffer (50 mM Tris/HCl, pH 7.3, 100 mM NaCl, 0.1% Triton X-100, 2 mM MgCl_2 and MnCl_2 , 10 mM β -glycerophosphate and NaF) containing 0.2 μg of peptide. After 10 min on ice, 1 μCi of [γ - ^{32}P]ATP together with 0.1 μM ATP and 0.2 μM poly-L-lysine were added. Tubes were incubated for exactly 5 min at room temperature and phosphorylation was stopped by heating the samples for 5 min at 95°C. The phosphorylated peptides were electrophoresed on thin-layer cellulose plates in acetic acid/pyridine/water (5/0.5/94.5, pH 3.5) for 90 min at 500 V. The plates were dried and autoradiographed. Spots corresponding to phosphorylated peptides were scraped and the radioactivity was quantitated by Cerenkov counting.

The phosphopeptide mapping was carried out as previously described (Mérèse et al., 1990).

Galactosyltransferase and sialyltransferase activities were determined as follows. For the galactosyltransferase, 20 μl of fractions were mixed with 80 μl of 0.1 M Tris/HCl, pH 7.3, 0.15 M NaCl, 0.5% Triton X-100, 10 mM MnCl_2 , 5 mM ATP, and 0.5 mg/ml ovomucoid as substrate. 1 μCi UDP-D-[6- ^3H]galactose was added. For the sialyltransferase, 20 μl of fractions were mixed with 80 μl of 50 mM NaCacodylate, pH 6.8, 0.5% Triton X-100, and 2.5 mg/ml asialofetuin. 0.1 μCi (5 μM) CMP- ^{14}C sialic acid were added. The reactions were carried out for 1 h at 37°C. Substrates were precipitated with 10 vol of 5% phosphotungstic acid in 0.1 N HCl for 15 min on ice. Tubes were centrifuged for 15 min at 4°C in a table top centrifuge. Pellets were washed four times with 5% TCA, resuspended in 0.5% SDS, and counted.

Results

Phosphorylation and Transport of the CI-MPR in the Biosynthetic Pathway

To determine when the newly synthesized CI-MPR acquires phosphate groups on serines 2421 and 2492 of its cytoplasmic domain, we followed an approach (see Fig. 1) similar to that developed by Duncan and Kornfeld (1988) that takes advantage of the presence of the sialyltransferase acting on N-linked oligosaccharides in late Golgi compartments, namely the TGN. The essence of this approach is that acquisition of sialic acid on the oligosaccharides of the CI-MPR reflects its movement through this compartment. We also made use of the drug dMM, a specific and reversible inhibitor of the early Golgi α -mannosidase I, a key enzyme in the

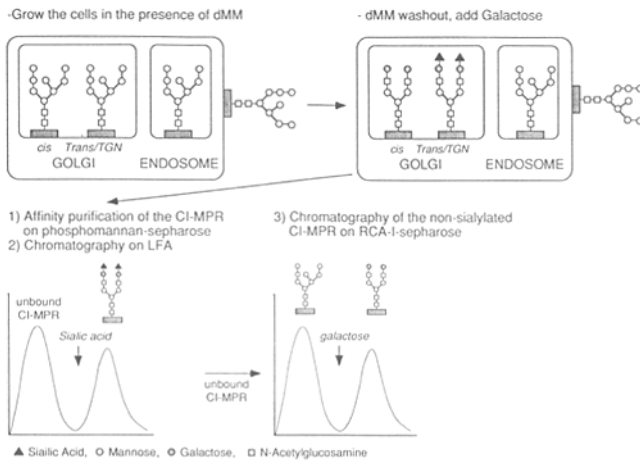


Figure 1. Experimental protocol used to follow phosphorylation of the newly synthesized CI-MPR in the biosynthetic pathway.

processing of the N-linked oligosaccharides (for review see Elbein, 1991). When cells are grown in the presence of this drug, cellular glycoproteins, including the MPRs contain only high-mannose N-linked oligosaccharides. Upon removal of the drug, the oligosaccharides of newly synthesized proteins are normally converted to sialylated complex-type oligosaccharides. Therefore, these newly synthesized, sialylated glycoproteins can be specifically retained on immobilized LFA, a lectin specific for sialyl residues (Goda and Pfeffer, 1988). Alternatively, the newly synthesized glycoproteins which are in transit in the galactosyltransferase-rich sialic acid residues can also be purified on immobilized RCA-I, a lectin specific for oligosaccharides with terminal galactose residues. Since the CI-MPR bears N- and O-linked oligosaccharides both containing galactose and sialic acid residues, we performed our study on the CHO ldl-D cells which are unable to add O-linked oligosaccharides to proteins and therefore allowed us to follow only the modifications occurring on N-linked sugar chains (Kingsley et al., 1986).

Thus, CHO ldl-D cells were grown in the presence of dMM. They were then labeled for 1 h with [³⁵S]methionine in the absence of the drug and in the presence of galactose to restore normal processing of N-linked sugars (see Fig. 2 A). The CI-MPR was affinity-purified on a phosphomannan matrix and applied onto the LFA lectin column. The receptor which did not interact with this lectin was further applied onto a RCA-I lectin column. Fig. 2 B shows that, after the labeling period, the newly synthesized CI-MPR was still located in the ER and/or the early Golgi since it did not interact with lectins specific for sialic acid and galactose residues. When the cells were chased for various periods of time in the presence of an excess of unlabeled methionine, the CI-MPR started to acquire sialic acid but with a lag time of 1 h. Most of the labeled CI-MPR was sialylated after 2–3 h of chase ($t^{1/2} \approx 1.5$ h). As expected, galactosylation of the newly synthesized receptor was observed slightly earlier than sialylation, reaching a maximum after 1–2 h of chase and decreasing slowly with further chase times. An identical experiment was performed with cells pre-labeled with

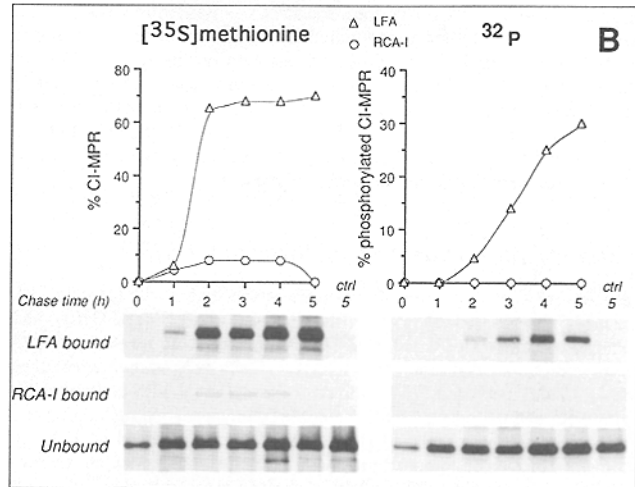
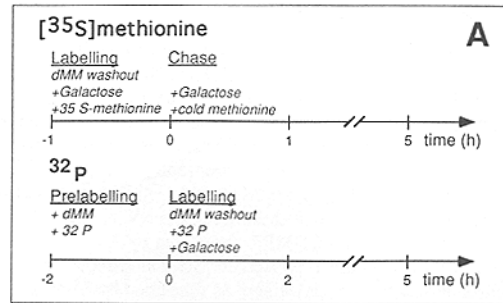


Figure 2. The newly synthesized CI-MPR is phosphorylated when it acquires sialic acid. (A) schematic protocols used for the labeling of ldl-D CHO cells. (B) ldl-D CHO cells were grown with dMM in low serum as described in Materials and Methods. (Left) The cells were incubated at 37°C without dMM but with galactose and labeled with [³⁵S]methionine. After 1 h of pulse, the cells were chased for various periods of time and the CI-MPR was purified by affinity chromatography on phosphomannan. The sialylated form of the CI-MPR was first retained on a LFA affinity column (△). The unretained galactosylated form of the CI-MPR was then purified on a RCA-I lectin column (○). The high mannose form of the CI-MPR (unbound fractions) as well as the sialylated and galactosylated CI-MPR were subjected to SDS-PAGE and the radioactive bands were quantitated by densitometric scanning. (Right) ldl-D CHO cells were grown with dMM and low serum and pre-labeled with [³²P]orthophosphate. After a 2 h period of prelabeling, the cells were incubated at 37°C without dMM but with galactose and [³²P]orthophosphate. After various periods of time for the dMM washout, the cells were harvested and the different forms of the CI-MPR were purified and analyzed as above. As controls (ctrl), the same experiments were carried out in the presence of dMM and in the absence of galactose.

[³²P]orthophosphate (Fig. 2 B). Immediately after the release of the dMM block (within the first hour), no phosphorylated CI-MPR could be retained on the LFA or on the RCA-I affinity columns. Since the newly synthesized CI-MPR bearing endo H-sensitive oligosaccharides is not phosphorylated in CHO cells (Sahagian and Neufeld, 1983), the unbound phosphorylated species must represent the endogenous CI-MPR also bearing high mannose oligosaccharides. The newly synthesized, sialylated CI-MPR which started to be efficiently retained on the LFA column 2 h after the dMM wash out, was phosphorylated. Then, the amount of phos-

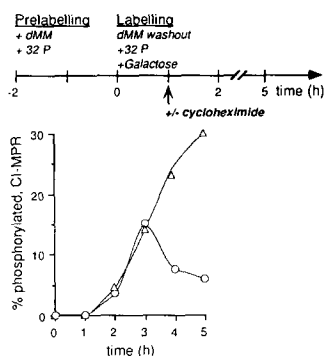


Figure 3. Phosphorylation and dephosphorylation of the newly synthesized CI-MPR. Irl-D CHO cells were prelabelled and labeled with [³²P]orthophosphate as described in the legend of Fig. 2 but cycloheximide was added 1 h after the dMM washout in order to block protein synthesis (○). As a control, subsequent incubations were performed without cycloheximide as in Fig. 2 (Δ). The CI-MPR was purified and the sialylated forms were analyzed as before.

phorylated, sialylated CI-MPR increased linearly with time due to its continuous synthesis. In contrast, the fraction of the CI-MPR which was not sialylated but retained on the RCA-affinity column was never phosphorylated (Fig. 2 B). When cycloheximide was added 1 h after the release of the dMM block in order to have a pulse of protein synthesis,

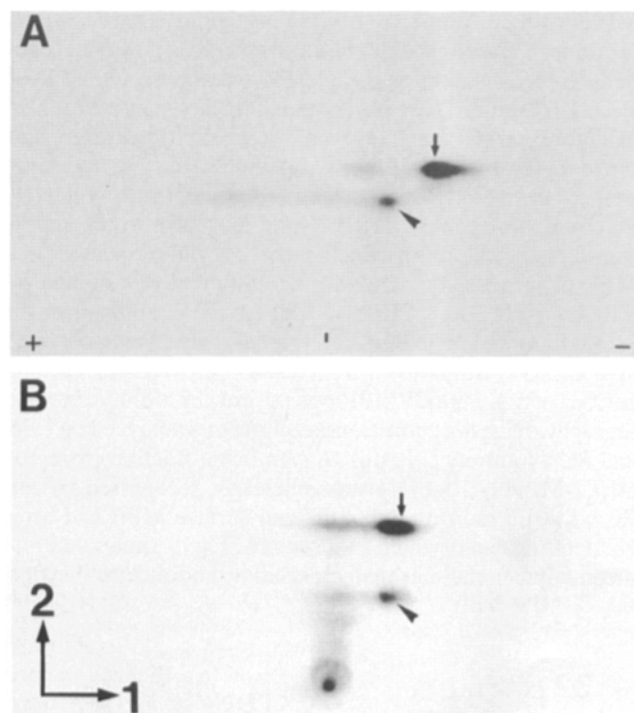


Figure 4. Analysis of CI-MPR phosphopeptides from CHO and MDBK cells. Irl-D CHO cells (B) were labeled with [³²P]orthophosphate as described in legend of Fig. 3. Cells harvested 2 h after addition of cycloheximide and the sialylated CI-MPR was purified. The phosphopeptides were separated in the first dimension by electrophoresis (1) and in the second dimension by chromatography (2). For comparison, MDBK cells (A) were labeled to equilibrium with [³²P]orthophosphate and the corresponding tryptic phosphopeptides were obtained from the immunoprecipitated receptor. The arrowhead corresponds to residues 2480–2499 of the full-length bovine CI-MPR precursor, the arrow to residues 2407–2432.

the amount of phosphorylated CI-MPR interacting with the LFA-lectin column reached a maximum 3 h after synthesis and then decreased rapidly with time (Fig. 3). Cycloheximide had no effect on the phosphorylation of the non-sialylated endogenous receptor (not shown). We conclude from these different results that phosphorylation does not occur when the CI-MPR moves through the *trans*-Golgi region. Phosphorylation and sialylation rather appear as two closely related events strongly suggesting that phosphorylation occurs when the CI-MPR is in the TGN or immediately after leaving this compartment. Our data also indicate that these phosphate groups are removed when the CI-MPR has reached a post-Golgi compartment, presumably endosomes.

The phosphorylated CI-MPR of CHO Irl-D cells and MDBK cells was analyzed by two dimensional phosphopeptide mapping after trypsin digestion. Fig. 4 confirmed that, in CHO cells the CI-MPR was effectively phosphorylated on similar sites as those seen in the bovine CI-MPR (serines 2421 and 2492). The stoichiometry of phosphorylation was then determined for the newly synthesized sialylated CI-MPR. Table I indicates that it contained ≈2 moles of phosphate per mole of CI-MPR. In contrast, the total pool of CI-MPR contained ≈0.17 moles of phosphate per mole of receptor, suggesting that, at steady state, only ≈10% of the cellular receptor is fully phosphorylated. We conclude from these results that most of the CI-MPR leaving the TGN is phosphorylated on these two serines and that this posttranslational modification appears as a major transient event.

Phosphorylation and Transport of the Cell Surface CI-MPR in the Endocytic Pathway

We then examined whether a similar modification could occur during endocytosis of the CI-MPR via clathrin-coated vesicles and its subsequent transport through the different endocytic compartments. The first approach taken, was to tag the cell surface CI-MPR by incubating CHO cells at 4°C with an anti CI-MPR antibody. After washing the cells and quenching the unreacted binding sites of the bound antibodies with the soluble luminal domain of the CI-MPR purified from bovine serum, the cells were warmed up to 37°C for various periods of time. The internalized CI-MPR was first followed by microscopy using a second fluorescently labeled antibody. Fig. 5 shows the internalization of the cell surface CI-MPR using this assay. After a brief incubation at 37°C (2 min), a discrete punctate pattern was observed, consistent

Table I. Stoichiometry of Phosphorylation of the CI-MPR

	CI-MPR	³² P incorporated	³² P/CI-MPR
	(μg)	(cpm)	(ratio)
Total CI-MPR (steady state)	1.5	1780	0.17
Newly synthesized sialylated CI-MPR	0.18‡	189	1.9

The stoichiometry of phosphorylation was calculated as indicated in Materials and Methods. Values represent means of duplicates.

* The amount of total CI-MPR was estimated by Coomassie blue staining and visual comparison with standards.

‡ The amount of [³⁵S]methionine labeled newly synthesized receptor was determined by densitometric scanning of the LFA eluted versus total CI-MPR.

§ and ¶ Specific radioactivities of the [³²P]orthophosphate were 1,900 and 555 cpm/pmole, respectively.

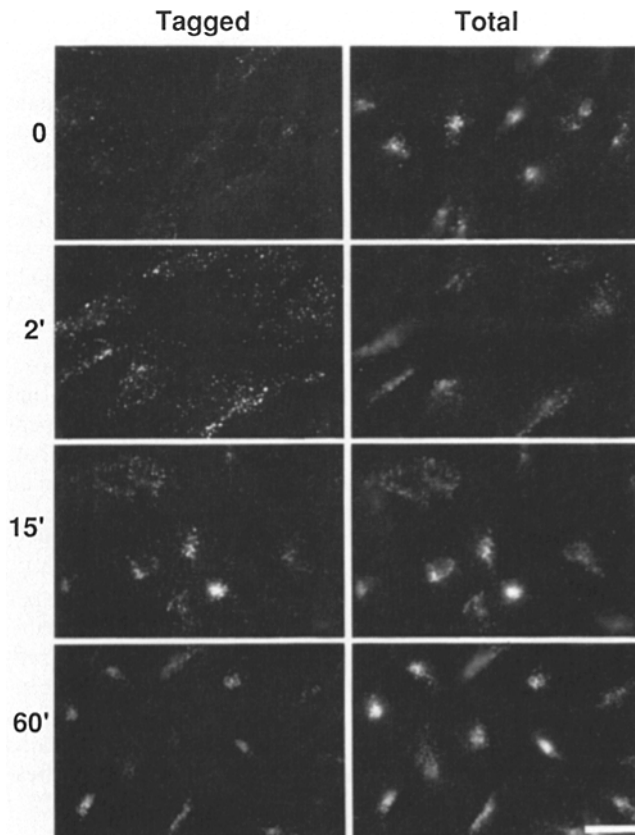


Figure 5. Fluorescence microscopy of endocytosis of the cell surface CI-MPR. CHO cells were incubated at 4°C with rabbit anti-CI-MPR antibodies as described in Methods to tag the cell surface CI-MPR. The cells were then reincubated at 37°C for the indicated periods of time. The intracellular (*Total*) and the cell surface/internalized (*Tagged*) CI-MPR were visualized by double immunofluorescence microscopy. Bar, 20 μ m.

with the notion that the cell surface CI-MPR had reached the early peripheral endosomes. With longer times of reincubation (15 min), the labeling became more concentrated in the perinuclear region and after 1 h the internalized receptor colocalized with the intracellular pool of receptor which is mostly found in late endocytic structures in many cell types (for review see Kornfeld and Mellman, 1989). This internalization process of the CI-MPR was repeated with cells

previously labeled to equilibrium with [³⁵S]methionine or [³²P]orthophosphate and the cell surface receptor was subsequently immunoprecipitated. For comparison, the intracellular, untagged CI-MPR was also immunoprecipitated. Fig. 6 shows that after binding of the anti CI-MPR antibody to the cell surface receptor at 4°C, \approx 10% of the [³⁵S]methionine-labeled receptor was immunoprecipitated, consistent with the surface distribution of the CI-MPR in many cell types. This cell surface CI-MPR was not significantly phosphorylated, a weak signal being observed only after long exposures. The amount of [³⁵S]methionine-labeled CI-MPR which could be immunoprecipitated with the pre-bound antibody did not change during the subsequent internalization at 37°C. This indicates that no intracellular receptor was coimmunoprecipitated along with the endocytosed CI-MPR. During short (2–5 min) or longer times (15–60 min) of internalization at 37°C, this endocytosed CI-MPR did not acquire phosphate groups. We interpret this result as indicating that the CI-MPR is not phosphorylated during its internalization from the cell surface or when it travels through the early and late endosomal compartments.

Phosphorylation and Recycling of the Cell Surface CI-MPR to the TGN

We then analyzed the phosphorylation of the cell surface CI-MPR during its return to the TGN using the assay described by Duncan and Kornfeld (1988). We made use of the exogenous galactosylation of the plasma membrane CI-MPR of Lec8 CHO cells. This mutant cell does not transport UDP-galactose inside the Golgi compartment (Deutscher and Hirschberg, 1986) and therefore synthesizes glycoproteins with N-linked oligosaccharides terminated with N-acetylglucosamine (Stanley, 1981). Upon its return to the sialyltransferase-rich compartment, the exogalactosylated CI-MPR acquires sialic acid and therefore can be retained by a LFA matrix. Lec8 CHO cells labeled to equilibrium with [³⁵S]methionine or [³²P]orthophosphate were galactosylated at 4°C. After subsequent reincubation at 37°C for various periods of time, the CI-MPR was purified by affinity chromatography on a phosphomannan column and analyzed on LFA and RCA-I affinity columns. A significant fraction of the total CI-MPR (\approx 10–15%) was efficiently recognized by the RCA-I lectin indicating that the cell surface MPR had been efficiently galactosylated (not shown). Fig. 7 shows that immediately after the exogalactosylation and during the first

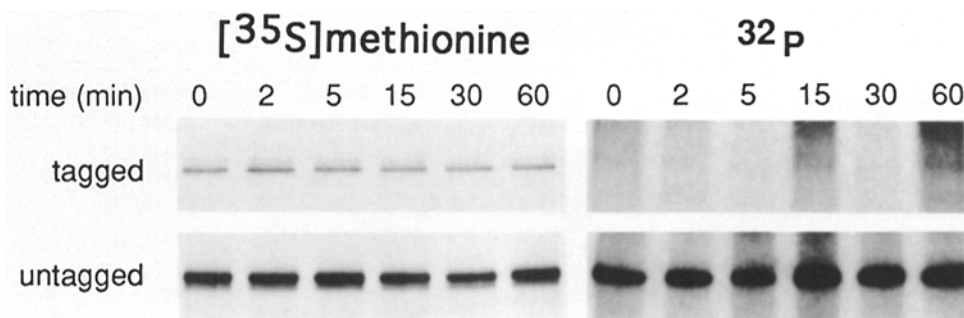


Figure 6. The cell surface CI-MPR is not phosphorylated during endocytosis. CHO cells labeled overnight to equilibrium with either [³⁵S]methionine or [³²P]orthophosphate were incubated for 1 h at 4°C with anti CI-MPR antibodies. After washing the excess of antibody and quenching with the soluble luminal domain of the CI-MPR (see Methods), the cells were

warmed up to 37°C and incubated for the indicated periods of time. The antibody tagged CI-MPR (*tagged*) was further immunoprecipitated and analyzed on SDS-PAGE. As a control, the endogenous untagged CI-MPR (*untagged*) was also immunoprecipitated.

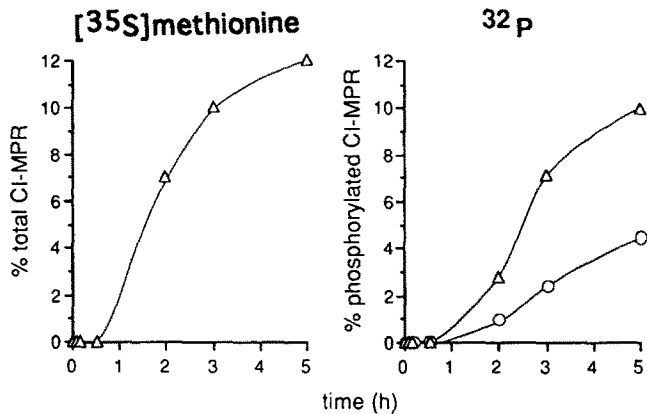


Figure 7. The cell surface CI-MPR acquires phosphate only when it had returned to the Golgi. Lec8 CHO cells were labeled overnight either with [³⁵S]methionine or with [³²P]orthophosphate. The cell surface oligosaccharides were then galactosylated at 4°C using purified galactosyltransferase as described in Materials and Methods. The cells were then warmed up to 37°C for the indicated periods of time. The CI-MPR was purified on a phosphomannan affinity column and the sialylated (Δ) and galactosylated (○) species were selected on specific lectins and analyzed by SDS-PAGE.

hour of reincubation at 37°C, no [³⁵S]methionine-labeled CI-MPR could be retained on the LFA column. In these conditions, no [³²P]labeled CI-MPR was retained by either column consistent with the idea that the cell surface receptor did not acquire phosphate groups during its recycling to the cell surface nor during its transport from the cell surface to endocytic compartments. However, after 2 h of reincubation at 37°C, a significant fraction of the exogalactosylated [³⁵S]methionine-labeled CI-MPR had acquired sialic acid residues and was retained on the LFA column. These kinetics of return to the TGN were similar to those described by Duncan and Kornfeld (1988). This sialylated CI-MPR was phosphorylated (Fig. 7). With long times (2–5 h) of reincubation at 37°C, we noticed that some galactosylated CI-MPR retained on RCA-I column also acquired phosphate, but nevertheless with similar kinetics as that of its sialylation. We interpret this later finding as indicating that some CI-MPR escapes resialylation due to a low content of sialyltransferase activity in these cells (Deutscher and Hirschberg, 1986). Therefore, we conclude from these results that the cell surface CI-MPR acquires phosphate only when it had returned to a sialyltransferase-rich compartment, namely the TGN.

Clathrin-coated Vesicles Contain Phosphorylated CI-MPRs

The MPRs and their ligands leave the TGN in clathrin-coated vesicles (Lemansky et al., 1987). Clathrin-coated vesicles were purified from labeled CHO cells using the method described by Woodman and Warren (1991). The last step of the fractionation procedure involves a centrifugation on a Ficoll/D₂O continuous density gradient. The clathrin-coated vesicles equilibrate in the dense bottom fractions whereas the Golgi, the plasma membrane and endosomes are recovered in the light top fractions (Fig. 8 A). The CI-MPR was immunoprecipitated from the fractions containing the

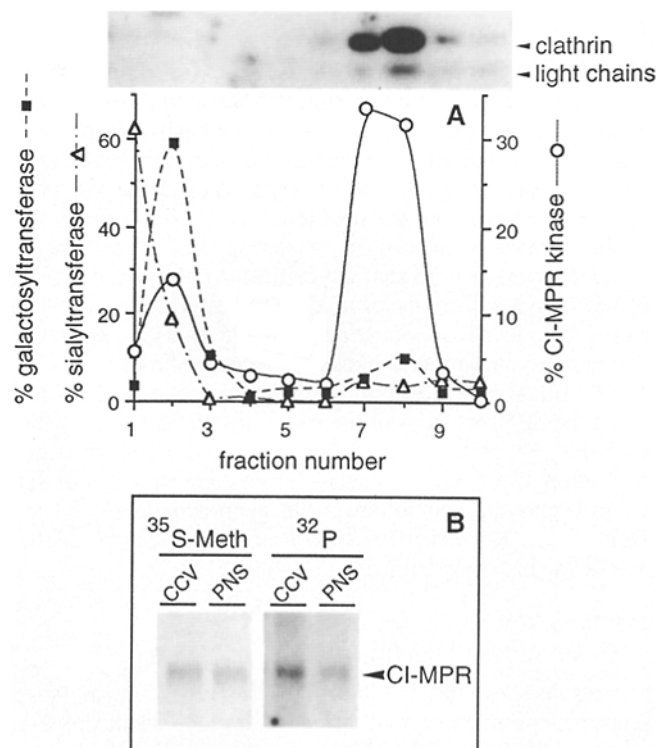


Figure 8. The phosphorylated CI-MPR and the corresponding CI-MPR kinase are found in clathrin-coated vesicles. (A) CHO cells were fractionated to separate clathrin-coated vesicles as described in Materials and Methods. The fractions were then analyzed for their content in galactosyltransferase (■), sialyltransferase (Δ) and membrane-associated CI-MPR kinase (○) activities. Results are expressed as percentage of the total membrane associated activity. The clathrin light chains were detected by western blotting (upper panel). (B) The same fractionation protocol was followed with CHO cells labeled overnight with [³⁵S]methionine or with [³²P]orthophosphate. Fractions 6–9 containing the clathrin-coated vesicles were pooled, solubilized with detergents, and the CI-MPR was immunoprecipitated (CCV, 12 μg of proteins) and analyzed on SDS-PAGE. For comparison, the CI-MPR was also immunoprecipitated from parts of the corresponding post-nuclear supernatants (PNS, 65 μg of proteins).

clathrin-coated vesicles and analyzed by SDS-PAGE. Fig. 8 B shows that the small proportion of the intracellular CI-MPR present in these vesicles (≈1% of the [³⁵S]methionine-labeled CI-MPR) was effectively labeled with [³²P]orthophosphate. We have previously shown that the kinase able to phosphorylate the serines 2421 and 2492 in the bovine CI-MPR tail is tightly associated with the Golgi-specific adaptor complex (Méresse et al., 1990). Therefore, we determined the distribution of this kinase activity throughout this density gradient. Fig. 8 A shows that most of the membrane-associated CI-MPR kinase activity (≈70%) was recovered in dense fractions containing the clathrin-coated vesicles with some (30%) remaining in the top fractions, presumably associated with the Golgi complex. Some kinase activity (60% of the total) was also detected as a soluble form in these top fractions (not shown). These results agree well with the notion that the CI-MPR is phosphorylated in the TGN and/or when it exits this organelle.

Discussion

The MPRs cycle constitutively between several cellular compartments. They must be transported between the TGN and endosomes in order to deliver newly synthesized lysosomal enzymes, and also between the plasma membrane and endosomes for the internalization of exogenous ligands. We have previously shown that the bovine CI-MPR cytoplasmic tail is phosphorylated *in vivo* on two serine residues present in two conserved regions that are critical for efficient delivery of newly synthesized lysosomal enzymes. Our two-dimensional tryptic phosphopeptide mapping analysis indicates that similar sites are also phosphorylated in the hamster CI-MPR tail. We have examined their phosphorylation state along the different steps of the recycling routes taken by the CI-MPR. For this, we have mostly made use of the compartmentation of the oligosaccharide processing enzymes in the Golgi apparatus and followed the approach developed by Duncan and Kornfeld (1988) to examine the return of the MPRs to this compartment.

Phosphorylation Occurs in the TGN or Golgi-derived Vesicles

Our results show that the CI-MPR exclusively acquires phosphate groups on the crucial serines of its cytoplasmic domain when it is present in the TGN and/or has just left this compartment. Phosphorylation and sialylation of the newly synthesized CI-MPR occur with very similar, if not identical kinetics. In contrast, the newly synthesized receptor is not modified on these serines when it passes through the *trans*-Golgi cisternae containing the galactosyltransferase. The CI-MPR also remains non-phosphorylated during its transport through the early biosynthetic pathway since the endo-H-sensitive form of the CI-MPR is not phosphorylated (Sahagian and Neufeld, 1983). Similarly, the CI-MPR originally present on the plasma membrane acquires phosphate groups on these sites only when it has been resialylated, *i.e.*, after it has returned to and/or has left a sialyltransferase-rich compartment. In contrast, the plasma membrane CI-MPR does not acquire phosphate groups when it enters plasma membrane clathrin-coated pits or when it reaches the early and late endosomes. Thus, this latter finding implies that the phosphorylation of a sialylated CI-MPR is closely associated with its sorting in the TGN and/or with its transport to endosomes.

Sorting of the newly synthesized lysosomal enzymes and their subsequent transport require the formation of clathrin-coated vesicles (Lemansky et al., 1987). Accordingly, we showed that phosphorylated CI-MPR is found in clathrin-coated vesicles. Since this receptor is not phosphorylated when it enters plasma membrane clathrin-coated pits, it seems reasonable to conclude that the phosphorylated CI-MPR must be present in the Golgi-derived vesicles. Our fractionation study to separate clathrin-coated vesicles from the Golgi complex also indicates that the corresponding kinase activity is predominantly associated with these transport vesicles, very little membrane-bound activity being recovered in fractions containing both the Golgi complex and endosomes. These results agree well with our previous studies showing that this CI-MPR kinase activity is enriched in clathrin-coated vesicles purified from brain and co-purifies with the Golgi-specific adaptor complex (Mésesse et al.,

1990). This would argue that the CI-MPR is phosphorylated *in vivo* during its transport from the TGN to endosomes. However, due first to the low abundance of CI-MPR in the TGN (<1% of the total in NRK cells; Griffiths et al., 1990) which must reflect its efficient sorting and second to the possibility of shearing artificially coated buds during homogenization, we cannot completely exclude the possibility that phosphorylation of the CI-MPR starts in the TGN and proceeds efficiently during its subsequent transport.

Finally, our stoichiometry data show that most of the CI-MPR is phosphorylated on the two critical serines of its cytoplasmic domain upon its departure from the TGN. Therefore, this event represents a major posttranslational modification occurring during this step of the CI-MPR recycling pathway. This major modification also appears to be transient: after being phosphorylated, the CI-MPR is rapidly dephosphorylated ($t^{1/2} \approx 1$ h). This result agrees with the turnover of phosphate groups on the CI-MPR in CHO cells (Sahagian and Neufeld, 1983). Since a phosphorylated receptor is found in clathrin-coated vesicles and transport from the TGN to endosomes is most likely rapid, our data strongly suggest that a phosphorylated CI-MPR is delivered to endosomes. The dephosphorylation of this receptor would occur later in these endosomal compartments. Therefore, it appears that phosphorylation/dephosphorylation of the CI-MPR tail is associated to its recycling between the TGN and endosomes.

Possible Implication for CI-MPR Trafficking

The two phosphorylated serines are contained in two conserved regions of the CI-MPR tail that are important for efficient intracellular retention of newly synthesized lysosomal enzymes (Lobel et al., 1989): mutant CI-MPRs lacking either the 40 or the 89 residues at their carboxyl terminal domain are unable to target efficiently lysosomal enzymes although they still function in endocytosis. However, several steps of the CI-MPR recycling pathway could be affected by these mutations: sorting in the TGN, retention in the endocytic pathway and recycling to the TGN. The precise role of these conserved regions is still not clear. However, our finding of their transient phosphorylation would strengthen the possibility that they are somehow involved in the intracellular routing of the CI-MPR. For example, phosphorylation of these serine residues could stabilize a putative sorting signal and thereby modulate its interaction with the Golgi-specific adaptors which have been proposed to interact with the CI-MPR cytoplasmic domain in a tyrosine-independent manner (Glickman et al., 1988). Alternatively, these phosphorylated domains of the CI-MPR tail could be recognized as an intracellular retention signal which would prevent this receptor from reaching the cell surface from the endosomes, a process that would facilitate the delivery of newly synthesized lysosomal enzymes to lysosomes. This notion would be consistent with the results obtained by Corvera and co-workers (1988) showing that, in H-35 hepatoma cells treated with insulin a decrease in the phosphorylation state of the cellular IGF II/CI-MPR was concomitant with an increase of its cell surface expression. If this latter possibility were correct, it would argue that retention of the CI-MPR in endosomal structures is not a passive process. This may require interaction with an additional sorting machinery or, alternatively the interaction between the CI-MPR tail and the

Golgi-specific adaptors which may persist when the complex has reached the endosomes. Several other proteins, including the CD-MPR and lysosomal glycoproteins are believed to follow the same clathrin-dependent pathway at the exit of the TGN. However, phosphorylation of these crucial sites would remain a peculiar feature of the CI-MPR. The cytoplasmic domains of the lysosomal glycoproteins (for review see Kornfeld and Mellman, 1989) do not contain similar phosphorylation sites in their short cytoplasmic domains. The CD-MPR which functions only partly in intracellular retention of lysosomal enzymes (Watanabe et al., 1991) and possibly in their secretion (Chao et al., 1991) contains a similar but not identical domain at its carboxyl terminus (Dahms et al., 1987; Pohlmann et al., 1987; Ma et al., 1991; Ludwig et al., 1992). Our data indicate that the CD-MPR tail is not phosphorylated (unpublished observations). If the two MPRs were packaged with the same efficiency into Golgi-derived vesicles, this would preclude phosphorylation as a signal for efficient sorting in the TGN and could indicate that this post-translational modification would only be involved in the next step of transport which may differ for the two MPRs. We are currently investigating these possibilities.

We are grateful to Ulrike Bauer for her excellent technical assistance and to our colleagues in the group for their critical comments. We wish to thank Jean Gruenberg, Gareth Griffiths, Thomas Ludwig, Kai Simons and John Toozé for critical reading of this manuscript.

S. Mérésse is a recipient of a European Economic Community fellowship, Science Plan, contract scl000316.

Received for publication 9 July 1992 and in revised form 16 September 1992.

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