Serine Phosphorylation Site of the 46-kDa Mannose 6-Phosphate Receptor Is Required for Transport to the Plasma Membrane in Madin-Darby Canine Kidney and Mouse Fibroblast Cells

Peter Breuer, Christian Körner, Christian Böker, Andrea Herzog, Regina Pohlmann, and Thomas Braulke*

Institute for Biochemistry II, Georg-August-Universität Göttingen, Gosslerstrasse 12d, 37073 Göttingen, Germany

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Up to 4% of the human 46-kDa mannose 6-phosphate receptor (MPR46) expressed in Madin-Darby canine kidney (MDCK) cells are localized at the cell surface. At steady state, the expression of MPR46 on the apical surface of filter-grown MDCK cells is about sixfold lower than on the basolateral surface. The cytoplasmic domain of the MPR46 is phosphorylated on serine 56 at low stoichiometry. By expressing mutant MPR46 we have shown that the MPR46 phosphorylation site is required for delivery to the plasma membrane. In addition, mutant MPR46 expressed in MPR-deficient mouse embryonic fibroblasts were not detected at the cell surface and their ability to sort newly synthesized cathepsin D was not altered. Since the loss of MPR46 phosphorylation correlates with the lack of cell surface expression, phosphorylation of serine 56 may either function as a direct plasma membrane targeting signal or inhibit MPR46 recycling from endosomes to Golgi, resulting in trafficking to the cell surface.

INTRODUCTION

Mannose 6-phosphate receptors (MPRs) segregate newly synthesized lysosomal enzymes from the secretory pathway and mediate their targeting to lysosomes. Two distinct homologous receptors with Mr, 300,000 (MPR300) and 46,000 (MPR46) have been found in mammalian cells differing in their expression level, ligand-binding properties, and transport functions (Kornfeld, 1992; Hille-Rehfeld, 1995). After MPR-dependent sorting of lysosomal enzymes in the trans-Golgi network (TGN), clathrin-coated vesicles were formed containing the receptor-ligand complexes. The transport vesicles fuse with an endosomal/prelysosomal compartment where ligands dissociate from MPRs due to the low pH. Lysosomal enzymes are then sorted and packed in the lysosomes while the receptors recycle to the Golgi complex to mediate further rounds of transport. Small fractions of both MPRs are transported to the plasma membrane where only the MPR300 mediates the endocytosis of both exogenous lysosomal enzymes and the nonglycosylated insulin-like growth factor II for delivery to lysosomes via the endocytic pathway. The MPR46 does not function in endocytosis of lysosomal enzymes (Stein et al., 1987b), which might be due to the slightly acidic pH required for efficient ligand binding. Both MPRs recycle from the plasma membrane to the TGN and exchange continuously with MPRs in all of the compartments (Braulke, 1996). In addition, the differential distribution of the two MPRs within the endosomal compartment (Klumperman et al., 1993) suggests distinct functions or sorting processes. The directed intracellular transport of the two MPRs along the biosynthetic and endocytic pathway is thought to be mediated by signaling elements which are proposed to be localized mainly in the cytoplasmic domains. Some of these signals which are required for efficient sorting of both MPR46 and MPR300 in the TGN and endocytosis from the plasma membrane have been identified (Johnson

* Corresponding author.
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Netherlands). Purchased were suberate dyl from Amersham (Buchler, Germany). Madin-Darby canine kidney (MDCK) cells and the human colon carcinoma-derived cell line Caco-2 are used as models for studies on membrane protein targeting in polarized cells. Thus, newly synthesized proteins either were sorted in the TGN for delivery directly to the apical or basolateral domain or indirectly to the basolateral membrane following by endocytosis and transcytosis to the apical membrane (Matter and Mellman, 1994).

Even though the same signal structures required for sorting in the TGN or endosomes in nonpolarized cells appear to be important for membrane proteins in polarized cells, it is unclear whether additional structural elements are needed for the selective transport to apical and basolateral membrane domains. One of the best studied membrane proteins in polarized cells, the polymeric immunoglobulin receptor (pIgR) contains a 17-residue segment (R653–R669) that is necessary and sufficient for basolateral delivery (Casanova et al., 1991). Two tyrosine-based signals (Y688 and Y734) and the phosphorylation of serine 726 regulate the internalization of pIgR (Okamoto et al., 1992, 1994). Finally, phosphorylation of serine 664 stimulates in the absence of receptor ligands the transcytosis of pIgR (Casanova et al., 1990; Hirt et al., 1993) by inactivation of the basolateral-sorting signal.

We have previously reported that the MPR46 is phosphorylated at serine 56 in its cytoplasmic domain by a casein kinase-2 (CK-2) like kinase (Hemer et al., 1993; Körner et al., 1994). In the present study, we investigated the role of phosphorylation for cellular distribution and polarized expression of MPR46. At steady state, about 3% of human MPR46 expressed in MDCK cells are phosphorylated. This correlates with the amount of receptors localized at the cell surface. Mutation of the phosphorylation site prevented the transport of MPR46 to the plasma membrane in MDCK and MPR-deficient mouse embryonic fibroblasts (MEFs) and the cycling between TGN and endosomes was not affected.

**Experimental Procedures**

\[^{[35]}S\]Methionine, \[^{[32]}P\]orthophosphate, and Na\[^{[25]}I\] were purchased from Amersham Corp. (Buchler, Germany). Iodo-Gen, disuccinimidyl suberate (DSS), and a Super Signal chemiluminescence system were purchased from Pierce Chemical Co. (Baound Beijerland, the Netherlands). Oligonucleotides were synthesized on an Applied Biosystems model 381 A solid-phase synthesizer.

A rabbit antiserum and an affinity-purified goat IgG against human liver MPR46 (Stein et al., 1987a) and the monoclonal anti-MPR46 antibody (21D3) have been described previously (Chao et al., 1990). A donkey anti-goat IgG coupled to Texas Red was obtained from Dianova (Hamburg, Germany). The 21D3 antibody was iodinated with the aid of Iodo-Gen to a specific activity of 2.5–3.4 \(\mu\)Ci/\(\mu\)g. Restriction enzymes, \(T_{4}\) DNA ligase, and \(Taq\) DNA polymerase were purchased from Promega (Madison, WI), Boehringer Mannheim (Germany), and Pharmacia Biotech (Freiberg, Germany).

**DNA Constructs**

Mutation of serine 35, serine 56 in the cytoplasmic domain of the human MPR46 (corresponding to residues 246 and 267 of the full-length human MPR46), and both serine 35/56 to alanine were those as previously described (Hemer et al., 1993). Mutation of serine 56 to aspartic acid was performed by polymerase chain reaction-assisted in vitro mutagenesis. The 5′ primer 5′-TGT TCA CCA G AGATCT C CCA CCTCA GT-3′ encompassed a BglII site (corresponding to the nucleotides 690–695 of the full-length human MPR46 cDNA; the BglII site is underlined). The oligonucleotide 5′-TAA GGATCC AT CTA C ATT GGT AAT AAA TGG TCA TCC TCT TCGTCC TCC-3′ served as a mutagenic 3′ primer (the mismatched nucleotides of a KpnI site are underlined; the stop codon is indicated by a broken line, and the replaced codon corresponding to serine 56 is boldface). The PCR product was digested with BglII and KpnI (corresponding to nucleotides 690 and 3 nucleotides after the amber codon) and cloned into the full-length MPR46 cDNA in pBHE. The mutation was verified by sequencing the final product. A mutant MPR46 with a truncation in the cytoplasmic tail (stop 23 corresponding to asparagine 234) was kindly provided by K. Denzer (this Institute). This mutant is described in detail elsewhere (Bresciani, Denzer, Pohllmann, and von Figura, unpublished data).

**Cell Culture**

MDCK cells were transfected with 15 \(\mu\)g of the pBHE plasmid and the pSV2 neo (G418 resistance) plasmid (15:1) using the calcium phosphate technique. Selection was performed with 1 \(mg/ml\) G418 in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) and penicillin/streptomycin. The MDCK cells were routinely grown on 35-mm dishes (Greiner, Germany) and 24-mm Transwell (Costar, Cambridge, MA) polycarbonate filters. MEFs lacking both MPR300 and MPR46 (MPR–/–; SV 23/1; Pohllmann et al., 1995) were transfected with 15 \(\mu\)g of the pBHE plasmid and the pGK-hygro (hygromycin B resistance) plasmid (15:1). Selection was performed with 0.1–0.5 mg/ml hygromycin B in MEM with 10% (vol/vol) FCS. Stable colonies were isolated and screened for MPR46 expression (Hemer et al., 1993). MEFs lacking MPR300 alone were those described previously (Pohllmann et al., 1995).

**Metabolic Labeling and Immunoprecipitation of MPR46**

Cells were labeled with \[^{[35]}S\]methionine (40 \(\mu\)Ci/ml) for 16 h or with \[^{[32]}P\]orthophosphate (0.5 mCi/ml) for 4 h followed by immunoprecipitation of MPR46 and analysis by SDS-PAGE and fluorography/autoradiography as described (Hemer et al., 1993). For determination of the life span of the cells were labeled with \[^{[35]}S\]methionine (200 \(\mu\)Ci/ml) for 1 h and chased for up to 72 h followed by immunoprecipitation. The determination of the stoichiometry of MPR46 phosphorylation was performed as described (Setton, 1991) with minor modifications. In brief, 60-mm dishes of MDCK cells expressing wild-type MPR46 were incubated for a total of 72 h in 2.5 ml of 90% methionine-free MEM, 8% MEM, and 2% dialyzed FCS with either 0.2 mCi of \[^{[35]}S\]methionine or 2 mCi of \[^{[32]}P\]orthophosphate with one change of labeling medium after 36 h. The MPR46 was immunoprecipitated from cell lysates and analyzed.

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were solubilized. Vol. 8, Indirect immunofluorescence to SDS-PAGE 120 containing 35S-labeled [35S]methionine at 4°C (7.5% acrylamide) 2 h. The reaction was stopped by the addition of 20 mM ethanolamine. The immunoprecipitated MPR46 was subjected to SDS-PAGE (7.5% acrylamide) and fluorography.

Cross-Linking of MPR46
Cells grown on 35-mm plates were metabolically labeled with [35S]methionine and permeabilized in 50 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.5) containing 150 mM NaCl, 5 mM β-glycerophosphate, 0.5% bovine serum albumin, and 0.25% saponin for 2 h at 4°C with three changes of buffer. The permeabilized cells were washed three times with 0.1 M HEPES buffer (pH 7.6) containing 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, and 8 mM glucose (buffer A) followed by incubation with 0.5 mM DSS in buffer A at 4°C for 15 min. The reaction was stopped by the addition of 20 mM HEPES buffer containing 0.1 M NaOH.

RESULTS
Expression and Stoichiometry of Phosphorylation of MPR46
To examine whether phosphorylation of MPR46 is required for efficient routing in epithelial cells, MDCK cells stably expressing wild-type (WT) human MPR46 and mutant MPR46 with serine substitutions and a truncated form (stop 23) of the cytoplasmic tail (Figure 1) were analyzed. Immunoprecipitation of MPR46 from cells metabolically labeled for 16 h revealed that the synthesis rate and processing of human MPR46 forms were comparable (Figure 2). Very weak reactivity of the antiserum or none at all was observed with endogenous MPR46 from nontransfected MDCK cells. These data were confirmed in permeabilized cells by indirect immunofluorescence staining of MPR46 in transfected MDCK cells that also did not differ in intracellular distribution. The calculated half-lives for WT MPR46 (31 ± 1 h) immunoprecipitated from MDCK cells pulse labeled for 1 h with [35S]methionine and chased for up to 72 h did not significantly differ from those of mutant MPR46, with a mean of 36 ± 2 h (range, 31–40 h; n = 2–4).

By labeling WT MPR46-expressing MDCK cells for 72 h with [32P]orthophosphate and [35S]methionine of known specific activity, the stoichiometry of phosphorylation of MPR46 was estimated to be 0.03 mol of phosphate/mol of receptor. Thus, although the MPR46 was phosphorylated at one site, the data indi-
cate that about 3% of total MPR46 are phosphorylated at equilibrium. Neither the serine 56 substitution mutants (SS56A, SS56D, and DS35/56A) nor MPR46-SS35A were phosphorylated in MDCK cells (our unpublished observations). These results are consistent with the findings in BHK cells expressing the SS35A mutant (Hemer et al. 1993), showing that serine 35 is required for efficient MPR46 phosphorylation at serine 56.

**MPR46 Sorting to the Plasma Membrane**

By immunofluorescence staining of nonpermeabilized MDCK cells, WT and the truncated (stop 23) MPR46 were found at the plasma membrane (Figure 3). Nontransfected cells and MDCK cells expressing the serine → aspartic acid substitution mutant (SS 56D) as well as the serine → alanine substitution mutants (SS35A, SS56A, and DS35/56A) showed no antibody immunoreactivity at the plasma membrane.

To confirm the cellular distribution of human MPR46, binding of the 125I-labeled monoclonal MPR46 antibody 21D3 was analyzed at 4°C in the presence or absence of 0.1% saponin. While in MDCK cells expressing the WT and the MPR46 stop 23 mutant, 1.6 ± 0.2 and 4.3 ± 1.5% of the total receptors (n = 4) are localized at steady state at the cell surface, no binding of the antibody at plasma membranes was detected in nontransfected MDCK cells or in cells expressing MPR46 mutants with substituted serine residues. These data were confirmed by sequential immunoprecipitation from [35S]methionine-labeled MDCK cells expressing WT MPR46, indicating the presence of 3–4% of the receptors at the cell surface (our unpublished results). By selective immunoprecipitation of MPR46 from the cell surface of 32P-labeled MDCK cells, no increase in relative 32P incorporation compared with receptors from intracellular membranes was observed.

Due to the low MPR46 expression at the cell surface, the cycling of receptors via the plasma membrane was measured by the more sensitive endocytosis assay in which the uptake of the 125I-labeled 21D3 antibodies during an incubation at 37°C is measured. Figure 4 shows that the amount of endocytosed 125I-labeled antibodies in MDCK cells expressing the WT MPR46 increased with the time of incubation. No degradation of endocytosed 125I-labeled 21D3 was detectable during the 3-h incubation period.

In MDCK cells the uptake of 21D3 antibodies followed a saturation curve with dependence on MPR46 expression. If the relative expression of WT MPR46 was higher than 5 ng of 21D3/mg cell protein, no alteration in the absolute amount of endocytosed antibodies was observed under our conditions (Figure 5). This indicates that parameters other than expression are rate limiting for MPR46 recycling. In nontransfected MDCK cells, no endocytosis of 21D3 antibodies was observed at all.

From data of a previous study, it was suggested that the MPR46 exists in different oligomeric forms depending on its subcellular localization (Waheed and von Figura, 1990). To determine whether the quaternary structure of mutant MPR46 is altered, radiolabeled semi-intact cells were cross-linked with the noncleavable reagent DSS. After cross-linkage, MPR46 were immunoprecipitated and analyzed using SDS-PAGE. The WT and mutant MPR46 exist in membranes mainly as a dimer (ratio of dimer:monomer MPR46 = 4:1). No immunoreactive bands corresponding to the MPR46 tetramer were detected (Figure 6).

**Polarized Surface Expression of MPR46**

To determine the polarized distribution, the binding of 125I-labeled 21D3 antibodies to the apical or basolateral side of filter-grown MDCK cells expressing the WT MPR46 was measured at 4°C. The results were normalized by antibody binding to nontransfected MDCK cells. About five- to sixfold more 21D3 antibody bound at steady state to the basolateral than to the apical cell surface (n = 5). The uptake of antibodies at 37°C was about threefold higher from the basolateral than from the apical medium of MDCK cells expressing the WT MPR46 (Figure 7). No endocytosis of antibodies neither from the apical nor basolateral domain was detected in nontransfected or MDCK cells expressing nonphosphorylated MPR46 mutants.

**Expression of MPR46 Mutants in MPR-deficient Mouse Fibroblasts**

To rule out the possibility of misrouting MPR46 transfected in MDCK due to the expression of both endog-
Figure 3. Immunofluorescence staining of WT and mutant human MPR46 in nonpermeabilized MDCK cells. Cells were grown on plastic dishes and fixed with 3% paraformaldehyde 2 d after plating. Cells were then incubated with preabsorbed antihuman MPR46 polyclonal IgG followed by Texas Red-conjugated goat anti-rabbit antibody and viewed under identical conditions. Bars, 10 μm.

DISCUSSION

The results presented in this article demonstrate that the MPR46 is transported to the apical and basolateral plasma membrane in polarized MDCK cells. The delivery to the plasma membrane requires the serine 56 phosphorylation site in the cytoplasmic domain of MPR46. We have also shown that MPR46 phosphorylation is not essential for the sorting of newly synthesized cathepsin D in the TGN.
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Figure 4. Time course of anti-MPR46 antibody uptake. Nontransfected (○) and WT (●) expressing MDCK cells were incubated for various times with 125I-labeled 21D3 antibodies (250,000 cpm/plate) at 37°C. Cell surface-bound antibodies were then displaced by the acid wash procedure at 4°C, and the endocytosed antibodies were quantified. The values are given as means ± SD of three independent experiments.

Figure 5. Uptake of 21D3 receptor antibodies in MDCK cells expressing WT or mutant MPR46. Transfected MDCK cells differing in the relative expression of WT or mutant MPR46 were incubated with 125I-labeled 21D3 antibodies at 37°C. After 3 h the cells were chilled to 4°C, and the endocytosed 125I-labeled 21D3 receptor antibodies were estimated following the removal of cell surface-associated 125I-labeled 21D3 at pH 2.5. The values were corrected for degradation of endocytosed ligands. ○, WT MPR46; ●, MPR46-SS535A; ■, MPR46-SS56A; □, MPR46-DS35/56 A; ▲, MPR46-SS56D.

Semiquantitative analysis of immunogold-labeled cryosections and immunoblot analysis of subcellular fractions of different cell types indicate that 1) the majority of the two MPRs codistribute in intracellular membranes of the TGN, endosomes, and cytoplasmic vesicles; 2) 5–10% of the total MPRs are localized at the cell surface within the same coated pits; and 3) the MPR46 is enriched over the MPR300 in associated tubules and vesicles of both early and late endosomes (Bleukemolen et al., 1988; Messner et al., 1989; Griffiths et al., 1990; Klumperman et al., 1993). Contradictory results have been reported on the MPR localization at the plasma membranes of polarized cells. Apical localization of MPR300 was shown in the Caco-2 cell line, rat retinal pigment epithelial cells, and proximal tubule cells of rat kidney (Tarnowski and McLaughlin, 1988; Klumperman et al., 1992; Cui et al., 1993). In MDCK cells, however, the MPR300 was found to be exclusively on the basolateral surface whereas the MPR46 could not be detected either apically or basolaterally (Prydz et al., 1990). The binding of the monoclonal MPR46 antibody 21D3 showed an approximately sixfold higher expression level of human MPR46 on the basolateral surface compared with the apical domain of MDCK cells. Because both the transport kinetics of MPR46 cycling via apical and basolateral membranes and their transcellular transport rates are unknown, it is not possible to conclude from the present data whether the MPR46 is preferentially transported to the basolateral surface. Since MPR46 present at the cell surface is not capable of binding and uptake of lysosomal enzymes (Stein et al., 1987b), its function at the cell surface is still unclear.

Several signal structures that mediate the directed intracellular transport have been localized in the cytoplasmic domain of MPR46. A carboxyl-terminal dileucine motif is essential for sorting of lysosomal enzymes in the Golgi and two signals are necessary for rapid internalization at the cell surface, requiring both a phenylalanine- and a tyrosine-based motif (Johnson et al., 1990; Johnson and Kornfeld, 1992b). Recently, a third signal was described consisting of six amino acids in the middle part of the cytoplasmic domain which prevents lysosomal degradation of MPR46 (Rohrer et al., 1995). From studies with several transmembrane proteins, basolateral-sorting signals have been identified which reside either within the transmembrane or luminal domain (Roman and Garoff, 1986) or in the cytoplasmic domain overlapping with the endocytic signal or are separate from such a signal (for review, see Matter and Mellman, 1994). For example, two signals that require both a tyrosine and a cluster of acidic residues function in basolateral delivery of the low-density lipoprotein receptor which belongs to the latter group (Matter et al., 1992). Another member of this group, the plgR, contains an autonomous 14-amino acid signal that is essential for the direct transport from the TGN to the basolateral membrane as well as for recycling from endosomes to the cell surface (Aroeti and Mostov, 1994). The data presented here indicate that the serine 56 phosphorylation site in the cytoplasmic domain of MPR46 that is also localized in a cluster of acidic residues serves as a plasma
Although substitution linked MPR46 mass marker standards in the presence and absence of 0.5 mM DSS for 15 min at 4°C. The receptors were immunoprecipitated and analyzed using SDS-PAGE (7.5% acrylamide) and fluorography. The positions of the cross-linked MPR46 dimer (D) and monomer (M) are indicated. Molecular mass marker standards are indicated on the left.

Figure 6. Cross-linkage of WT and mutant MPR46 in MDCK cells. (125I]Methionine-labeled cells were permeabilized and incubated in the presence and absence of 0.5 mM DSS for 15 min at 4°C. The receptors were immunoprecipitated and analyzed using SDS-PAGE (7.5% acrylamide) and fluorography. The positions of the cross-linked MPR46 dimer (D) and monomer (M) are indicated. Molecular mass marker standards are indicated on the left.

A mutant receptor containing alanine in place of the unique phosphorylation site serine 56 fails to be transported to the plasma membrane. Although substitution of serine 35 with alanine reduced the MPR46 phosphorylation efficiency in BHK cells (Hemer et al., 1993), this mutation completely prevented phosphorylation of MPR46 in MDCK cells. Application of the turns prediction hypothesis (Chou and Fasman, 1978; Rose, 1978) to the amino acid sequence of the cytoplasmic domain of MPR46 revealed that serine 35 is located in a β-turn structure which might be important for efficient receptor phosphorylation. Thus, the correlation between loss of phosphorylation and the lack of MPR46 expression at the cell surface suggests that phosphorylation of serine 56 is required for transport to the plasma membrane. This result is consistent with data on plgR that have shown that mutation of the serine 726 phosphorylation site reduces the number of receptors at the basolateral surface and subsequently the rate of dimeric IgA transcytosis (Hirt et al., 1993). Additionally, the mutant receptor exists as a monomer, and the authors speculate that serine 726 phosphorylation might trigger receptor dimerization required for proper basolateral sorting. Our cross-link experiments, however, showed that the MPR46 dimerization was not affected by serine 56 mutation, suggesting the involvement of another mechanism in the receptor-

Figure 7. Polarized uptake of 21D3 receptor antibodies. Filter-grown MDCK cells expressing WT or mutant MPR46 were incubated with 125I-labeled 21D3 antibodies in the apical (C) or basolateral (I) medium for 3 h at 37°C. The endocyted antibodies were determined as described in Figure 5. The results represent the means ± SEM of 5–15 experiments.

Figure 8. Uptake of 21D3 receptor antibodies in MPR-deficient MEFs expressing WT and mutant MPR46. WT or mutant MPR46 expressing MPR-deficient MEFs were incubated with 125I-labeled 21D3 antibodies for 3 h at 37°C. The cell-associated radioactivity was determined and related to cell protein (mean ± SD, n = 3). Uptake was corrected by the unspecific cell-associated radioactivity in MPR−/− cells. The levels of MPR46 expression in the transfected cell lines are expressed relative to the WT MPR46 cell line: MPR46-SS35A, 0.5 and MPR46-SS56D, 1.4.

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that phosphorylation affects the percentage of small vesicles containing ceramide and the identity of the 65-kDa protein is unprocessed procathepsin D (Pro CD) and the mature cathepsin D are indicated. The percentage of cathepsin D sorted is listed below each cell line. The identity of the 65-kDa protein (*) coprecipitated from the media by the CD antiserum was determined using SDS-PAGE and fluorography (representative of five experiments). The positions of the unprocessed procathepsin D and the mature cathepsin D are indicated.

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