The 46 kDa mannose-6-phosphate receptor contains a signal for basolateral sorting within the 19 juxtamembrane cytosolic residues

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The cytosolic domain of the 46 kDa mannose-6-phosphate receptor (MPR 46) contains a signal that mediates sorting of the receptor and of a reporter protein to the basolateral surface domain of Madin–Darby canine kidney cells. Progressive truncation of the 67 cytosolic residues indicated that the 19 juxtamembrane residues are sufficient for basolateral sorting. Alanine/glycine-scanning mutagenesis identified Glu-11 and Ala-17 as the critical residues between residues 7 and 19. Glu-11 is also of critical importance for the one of the three internalization signals in the cytosolic tail of the receptor [Denzer, Weber, Hille-Rehfeld, von Figura and Pohlmann (1997) Biochem. J. **326**, 497–505]. Although overlapping, the signals for basolateral sorting and internalization depend on different residues. The basolateral sorting signal of MPR 46 is distinct from tyrosine- or dileucine-based basolateral sorting signals and also lacks similarity to the few other basolateral signals that do not fall into these two classes.

INTRODUCTION

Epithelial cells are linked to each other by tight junctions, which block the diffusion of lipid and transmembrane proteins in the outer leaflet of the plasma membrane and represent the border between the apical and basolateral domain of the plasma membrane, facing the external and the internal milieu respectively. Epithelial cells possess mechanisms to distribute newly synthesized lipids and transmembrane proteins asymmetrically to the two domains and to maintain the compositional and functional asymmetry during the membrane traffic accompanying endocytosis and transcytosis (reviewed in [1–4]). In Madin–Darby canine kidney (MDCK) cells most newly synthesized plasma membrane proteins are sorted in the *trans*-Golgi network (TGN) into vesicles that are delivered directly either to the basolateral or to the apical domain [5–9]. Transmembrane proteins of the basolateral domain carry their sorting information in the cytosolic domain [10–23].

The signals for basolateral sorting fall into three groups. The largest group is represented by signals that contain a critical tyrosine residue. These signals are often overlapping with tyrosine-based signals that mediate internalization or targeting from the TGN to endosomes through coated vesicles. Replacement of the tyrosine residue can disrupt basolateral targeting and rapid internalization or sorting at the TGN, as shown for the lysosomal membrane glycoprotein lamp1 [10,24], the influenza virus haemagglutinin mutant Y 543 [11], the asialoglycoprotein receptor [13] and the nerve growth factor receptor [12]. When examined more closely, as for the low-density lipoprotein receptor [14] and lysosomal acid phosphatase (LAP) [16], it became apparent that the signals for internalization and basolateral sorting overlap but depend on different residues. It should be noted that not all basolateral sorting signals that resemble tyrosine-based internalization signals mediate rapid internalization, as has been shown for the vesicular stomatitis virus glycoprotein G [20,21]. Similarly not all tyrosine-based internalization signals mediate basolateral sorting [17,25].

A second group of basolateral sorting signals found in the IgG Fc receptor [22] and the hyaluronan receptor CD 44 [23] contains a dileucine motif. As with tyrosine-based signals, the dileucine-based signals are known to mediate sorting from the TGN to endosomes and rapid internalization [22]. The third group of basolateral sorting signals is heterogeneous; they lack similarity to each other or to the tyrosine- and dileucine-based signals. The group comprises the basolateral sorting signals in the polymeric immunoglobulin receptor [18], the transferrin receptor [20] and the dominant basolateral sorting signal in the low-density lipoprotein receptor [14,15].

Mannose-6-phosphate receptors (MPRs) are known to target newly synthesized lysosomal enzymes to lysosomes by their ability to cycle between the TGN, where the ligands are bound, and endosomes, where the ligands are released. In addition, MPRs cycle between the cell surface and endosomes. Although the cycling between endosomes and TGN and endosomes and plasma membrane is common to the two types of MPR known, only the larger of the two, with an apparent mass of 300 kDa (MPR 300), has the ability to internalize lysosomal enzymes. The smaller receptor, with an apparent molecular mass of 46 kDa (MPR 46), cannot bind lysosomal enzymes at the cell surface (reviewed in [26–28]). In MDCK cells, MPR 300 was detected exclusively at the basolateral domain [29], whereas in the intestinal adenocarcinoma cell line Caco-2 most of the surface receptor was associated with the apical villi [30].

In the present study we have analysed the polarity of MPR 46 in MDCK cells and identified the structural determinants for its basolateral distribution. The 19 juxtamembrane cytosolic residues were found to be sufficient for basolateral targeting. Alanine/glycine-scanning mutagenesis identified Glu-11 and Ala-

Abbreviations used: LAP, lysosomal acid phosphatase; mAb, monoclonal antibody; MDCK, Madin–Darby canine kidney; MPR, mannose-6-phosphate receptor; TGN, *trans*-Golgi network.

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17 as critically important residues, whereas none of the three aromatic residues (Phe-13, Phe-18 and Trp-19) was essential for basolateral sorting. This excludes the basolateral sorting signal of MPR 46 from categories that have homology with tyrosine- and dileucine-based sorting signals.

MATERIALS AND METHODS

Cell culture

MDCK cells (strain II) were grown in minimal essential medium supplemented with 10 % (v/v) fetal calf serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Polarized growth was achieved by plating 1.5×10^6 cells on 24 mm filters; experiments were performed 72 h after plating. For metabolic labelling and binding experiments, Transwell units (Costar, Cambridge, MA, U.S.A.) were used, and for indirect immunofluorescence experiments, Nunc transparent filter units (Nunc, Roskilde, Denmark) were used.

MPR 46 constructs

The construction of mutant MPR 46 cDNA species and the subcloning into the expression vector pMPSVHE was as described [24].

Stable transfection of MDCK cells

For stable expression, 1.5×10^5 MDCK cellper 6 cm dish were transfected with 20 μg of MPR 46 expression plasmid DNA and 0.5 μg of pSV2neo expression plasmid DNA, with the calcium phosphate technique [31]. After selection in medium containing 1 mg/ml G418 (Gibco BRL), resistant clones were tested for MPR 46 expression by binding of iodinated monoclonal antibody (mAb) 10C6 (see below). This antibody recognizes an epitope of the luminal domain of human MPR 46 [32].

Expression of MPR 46

To determine the expression level of MPR 46, confluent MDCK cells were incubated for 1.5 h at 4 °C in medium buffered with 20 mM Hepes/NaOH, pH 7.4, supplemented with 0.1 % saponin and iodinated mAb 10C6 (3×10^5 c.p.m./ml) as described [33]. For determination of MPR 46 at the cell surface, saponin was omitted from the incubation medium. When cell surface expression was determined in polarized cells the [^{125}I]-10C6 was offered either to the apical or to the basolateral side of the filters.

Metabolic labelling of MDCK cells and immunoprecipitation of MPR 46

Confluent MDCK cells or polarized MDCK cells were metabolically labelled for the indicated periods with [^{35}S]methionine (90 μCi/ml) in medium depleted of methionine. When polarized MDCK cells were used, [^{35}S]methionine was added to the basolateral chamber only. Cells were then either chased in MEM or chilled at 4 °C and washed with ice-cold PBS, scraped and pelleted for further analysis. The cells were resuspended in 10 mM Tris/HCl, pH 7.4, containing 0.5 M NaCl, 1 % (w/v) BSA, 0.1 % SDS, 1 % (w/v) sodium deoxycholate, 1.5 % (v/v) Triton X-100, 1 mM EDTA, 1 mM PMSF and 5 mM iodoacetamide (buffer A) and incubated overnight at 4 °C in the presence of a polyclonal antiserum recognizing both canine and human MPR 46. When MPR 46 was immunoprecipitated from MDCK secretions, media were mixed with 1 vol. of 2 × buffer A and incubated with the same antiserum as above. Immune complexes were pelleted and divided in two. One aliquot of each cell extract and medium was incubated overnight at 37 °C with 1 m-unit of endoglycosidase H [31]. Samples were then analysed by SDS/PAGE [10 % (w/v) gel] followed by fluorography.

Analysis of secretions

Polarized MDCK cells were metabolically labelled for 3 h with [^{35}S]methionine (90 μCi/ml). After a chase for 6 h the secretions from the apical and basolateral sides were collected. Secreted polypeptides were precipitated with $(NH_4)_2SO_4$ and, after dialysis against chromatography buffer, were subjected to MPR 300 affinity chromatography as described [34]. Polypeptides that were eluted with 5 mM mannose 6-phosphate were precipitated with trichloroacetic acid and analysed by SDS/PAGE [10 % (w/v) gel] followed by fluorography.

Indirect immunofluorescence

For cell-surface detection of MPR 46, polarized MDCK cells were washed from both the apical and basolateral sides with PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (DPBS) and fixed for 40 min with 3 % (w/v) paraformaldehyde in DPBS. Free aldehydes were then quenched by incubating samples for 10 min with 50 mM NH_4Cl in DPBS. Non-specific binding sites were blocked with three washes (10 min each) with DPBS containing 0.2 % gelatin; samples were then incubated for 1.5 h with the mAb 10C6 (20 μg/ml in 0.2 % gelatin in DPBS). After three washes with 0.2 % gelatin in DPBS, cells were incubated for 1.5 h in the presence of Texas Red-conjugated rabbit anti-(mouse IgG) (Sigma) diluted 1:300 in 0.2 % gelatin in DPBS. Excess of antibody was removed by 0.2 % gelatin washes; samples were then mounted in Mowiol. Analysis was performed with a Zeiss confocal laser scanning microscope. For intracellular distribution of MPR 46, MDCK cells grown on coverslips or on filters were permeabilized by treatment with 0.3 % Triton in PBS for 5 min before incubation with the antibody.

RESULTS

Sorting of MPR 46 to the basolateral plasma membrane in polarized MDCK cells

Human MPR 46 was stably expressed in MDCK cells. Expression was monitored by immunoprecipitation of metabolically labelled MPR 46, by binding of an iodinated monoclonal antibody (10C6) recognizing human but not canine MPR 46 and by indirect immunofluorescence (Table 1 and Figure 1).

On the basis of the incorporation of [^{35}S]methionine, the level of wild-type human MPR 46 expressed in MDCK cells was about 3-fold that of the endogenous MPR 46. In human MPR 46 two of the four N-linked oligosaccharides are processed to complex type structures, which are insensitive to endoglycosidase H [31]. The apparent size of the receptor and processing of N-linked oligosaccharides to endoglycosidase H-resistant oligosaccharides was similar for human and canine MPR 46, the human receptor seeming slightly larger (Figure 1). Indirect immunofluorescence with antibodies recognizing the canine and/or the human receptor revealed, in non-polarized MDCK cells grown in plastic dishes, a similar subcellular distribution of both with a prominent staining of perinuclear cisternae and vesicles distributed throughout the cytoplasm (results not shown). Binding of iodinated mAb 10C6 to non-permeabilized and cells permeabilized with 0.1 % saponin indicated that 1.3 % (0.6–2 %) of the MPR 46 was present at the cell surface (Table 1).

When the cells, grown on permeable filter supports to establish cell polarity, were fixed and exposed to mAb 10C6 from the basolateral side or both sides, only staining of the basolateral

Table 1 Expression, surface localization and carbohydrate processing of wild-type and truncated forms of transfected MPR 46 in MDCK cells

The binding values are means ± S.D. for five to fourteen independent determinations. All values were corrected for the binding of [^{125}I]-10C6 to control MDCK cells transfected with the vector only (399 ± 127 c.p.m. per 3 cm dish). The values in the surface receptor column represent [^{125}I]-10C6 bound to non-permeabilized cells as a percentage of antibody bound to permeabilized cells. Before calculation the values were corrected for binding to permeabilized control MDCK cells (209 ± 69 c.p.m. per 3 cm dish). Processing of oligosaccharides to complex type structures was estimated from the effect of endoglycosidase H (see the legend to Figure 1). Abbreviation: n.d., not determined.

MPR 46 form	Binding of [^{125}I]-10C6 (c.p.m. per 3 cm dish)	Surface receptor (% of total)	Processing of oligosaccharides
Wild-type	31321 ± 4974	1.3 ± 0.7	As endogenous
Stop 61	2744 ± 1439	8.9 ± 4.1	Decreased
Stop 50	2605 ± 1846	4.3 ± 3.6	Decreased
Stop 30	6308 ± 5165	3.4 ± 2.0	As endogenous
Stop 24	13091 ± 4337	8.2 ± 6.0	Decreased
Stop 20	2414 ± 1512	24.6 ± 14.1	Decreased
Stop 12	3194 ± 1304	13.9 ± 9.9	As endogenous
Stop 6	30914 ± 5660	5.7 ± 2.4	Decreased
Soluble	n.d.	n.d.	As endogenous

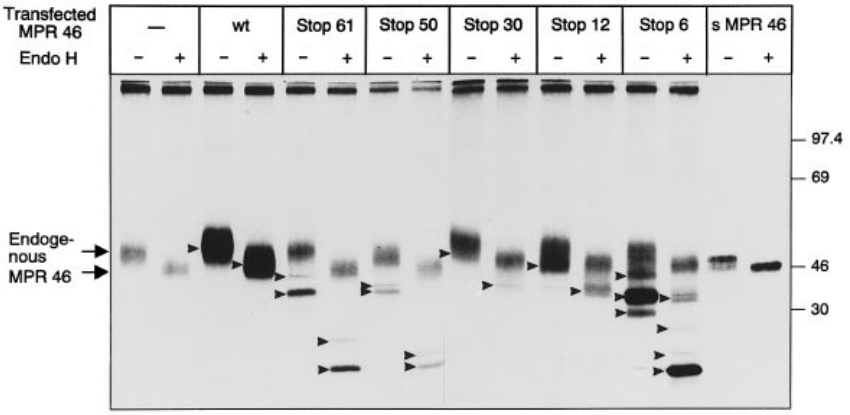

Figure 1 Expression of wild-type and truncated forms of MPR 46 in MDCK cells

MDCK cells transfected with vector alone or with the MPR 46 constructs indicated above the lanes were metabolically labelled with [^{35}S]methionine for 16 h. MPR 46 was immunoprecipitated from extracts of cells and media with a polyclonal antiserum recognizing endogenous canine MPR 46 and transfected human MPR 46. The solubilized immunoprecipitates were divided and one part was digested with endoglycosidase H. Shown are the immunoprecipitates of the cells except for soluble MPR 46 (sMPR 46), where the immunoprecipitates of the medium are shown. The position of canine MPR 46 before (upper arrow) and after digestion with endoglycosidase H (Endo H) (lower arrow) is indicated at the left margin. The positions of the transfected human MPRs are indicated by arrowheads. The positions of molecular mass markers are indicated (in kDa) at the right.

plasma membrane was observed. Horizontal sections obtained by confocal laser scanning microscopy revealed a honeycomb pattern (XY in Figure 2); sections perpendicular to the filter showed a columnar pattern reflecting the staining of the lateral membranes (Z in Figure 2). When the antibody was offered from the apical side, no staining was observed. Exclusive basolateral staining was also observed for a MDCK clone expressing MPR 46 at 40% of the wild-type level (results not shown).

The polarized distribution of MPR 46 was also evident when cells were incubated for 1.5 h at 4 °C in the presence of [^{125}I]-10C6. At the basolateral surface [^{125}I]-10C6 binding was 1816 ± 82 c.p.m. (determined in triplicate) and at the apical surface it was 467 ± 51 c.p.m. When these values are corrected for the non-specific binding of [^{125}I]-10C6 to the basolateral (220 ± 57 c.p.m.) or apical (269 ± 98 c.p.m.) surface of control MDCK cells, it is apparent that 90% of the binding sites for the antibody are localized at the basolateral membrane.

Because less than 2% of the receptor is expressed at steady state at the cell surface, we did not attempt to determine whether newly synthesized MPR 46 is transported directly to the basolateral domain or whether newly synthesized receptor is initially transported to both surface domains and polarity is achieved by subsequent redistribution from the apical to the basolateral domain.

To distinguish whether the MPR 46 detectable at the basolateral plasma membrane originates from the biosynthetic pool or is recycled to the surface from internal membranes, polarized MDCK cells were incubated for up to 8 h in the presence of 0.5 mM cycloheximide. This treatment abolished neither surface expression nor its polarity (results not shown), indicating that receptors at the surface originate from a receptor pool that recycles between internal membranes and the basolateral plasma membrane.

Overexpression of MPR 46 in non-polarized cells induces the secretion of newly synthesized lysosomal enzymes. This effect is thought to result from a competition of endogenous MPR 300 with MPR 46 for binding of newly synthesized ligands in the TGN. Because part of the ligands transported by MPR 46 become secreted, whereas those transported by MPR 300 are essentially all delivered to lysosomes, increased binding to MPR

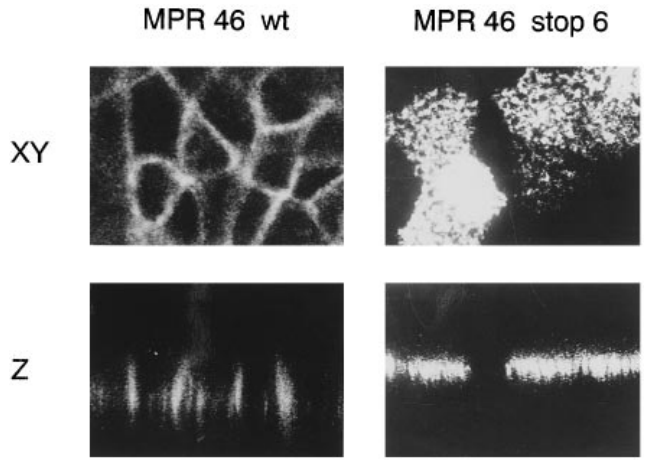

Figure 2 Cell-surface distribution of MPR 46 in polarized MDCK cells

Filter-grown MDCK cells expressing wild-type MPR 46 (MPR 46 wt) or a truncated form with a cytosolic tail of five residues (MPR 46 stop 6) were fixed without permeabilization and exposed to mAb 10C6 from both sides. Rhodamine-conjugated anti-(mouse immunoglobulin) was used as second antibody. Immunofluorescence was analysed with a confocal laser scanning microscope with a recording of a horizontal focal section (XY) through the middle (MPR 46 wt) or the apical (MPR 46, stop 6) region of the MDCK monolayer and of the vertical profiles (Z).

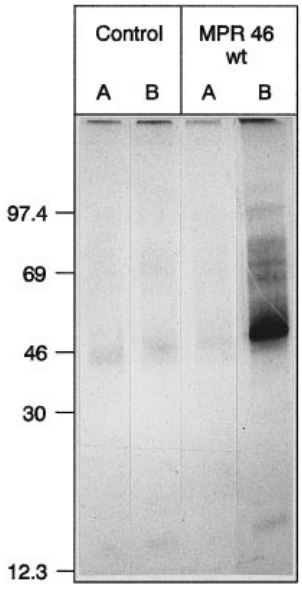

Figure 3 Expression of MPR 46 induces secretion of lysosomal proteins at the basolateral domain of the plasma membrane

Filter-grown MDCK cells transfected with vector alone (control) or an MPR 46 wild-type (wt) construct were metabolically labelled with [^{35}S]methionine for 3 h and chased for 6 h. The media from the apical (lanes A) and basal (lanes B) chambers were subjected to MPR affinity chromatography. Shown is the material that was eluted with 5 mM mannose 6-phosphate, then analysed by SDS/PAGE and fluorography. The positions of molecular mass markers are indicated (in kDa) at the left.

46 results in an increase in secretion [33]. To examine an induction of secretion of newly synthesized lysosomal proteins, filter-grown MDCK cells were metabolically labelled and the apically and basolaterally secreted polypeptides were examined for binding to an MPR 300 affinity matrix. The fraction of polypeptides that

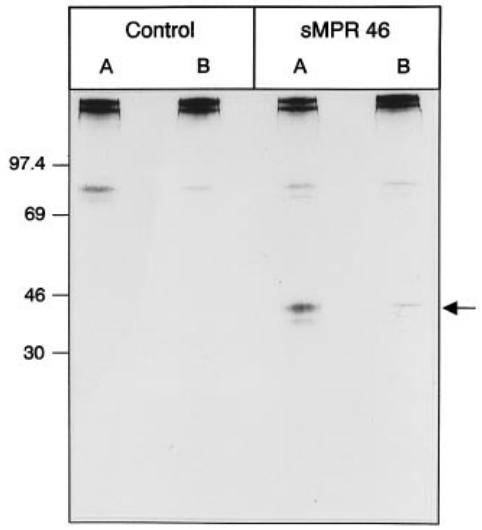

Figure 4 Soluble form of MPR 46 is secreted by polarized MDCK cells into the apical medium

Filter-grown MDCK cells were metabolically labelled with [^{35}S]methionine for 16 h and chased for 3 h. MPR 46 was immunoprecipitated from the apical (lanes A) and basolateral (lanes B) secretions of cells transfected with vector alone (control) or with a construct encoding a secretory form of MPR 46 (sMPR 46). Densitometric scanning indicated that 84% of sMPR (indicated by the arrow) is secreted apically and 16% basolaterally. The positions of molecular mass markers are indicated (in kDa) at the left.

were eluted with 5 mM mannose 6-phosphate was analysed by SDS/PAGE and fluorography. In MDCK cells transfected with the neomycin resistance gene and vector only, polypeptides that bound to MPR 300 were barely detectable in the apical or the basolateral medium (Figure 3, left panel). Overexpression of MPR 46 induced the secretion into the basolateral medium of a mixture of polypeptides that bound to the MPR 300 affinity matrix, and hence are considered as lysosomal proteins (Figure 3, right panel).

We conclude from these results that the transport of newly synthesized lysosomal proteins by MPR 46 includes passage to a basolateral compartment (early endosomes or plasma membrane), where the ligands dissociate and are released in part into the medium. Furthermore at steady state only a minor fraction of the receptor is expressed at the cell surface, where it is predominantly found at the basolateral domain and is recycled between the surface and internal membranes.

The cytosolic domain of MPR 46 is necessary and sufficient for sorting to the basolateral plasma membrane domain

When a truncated form of MPR 46 retaining only the five juxtamembrane residues of the 67 residues of the cytosolic domain of MPR 46 (MPR 46 stop 6) was expressed in MDCK cells, a small fraction of the receptor (5.7% of total; Table 1) was recovered at the cell surface. It should be noted that only a minor fraction of MPR 46 stop 6 acquired endoglycosidase H-resistant oligosaccharides (Figure 1), suggesting that the bulk of receptor does not exit from the endoplasmic reticulum. In polarized MDCK cells the stop 6 mutant was detected exclusively at the apical domain of the plasma membrane (Figure 2). These results indicate that the fraction of MPR 46 stop 6 that exits from the endoplasmic reticulum is sorted to the apical domain of the plasma membrane.

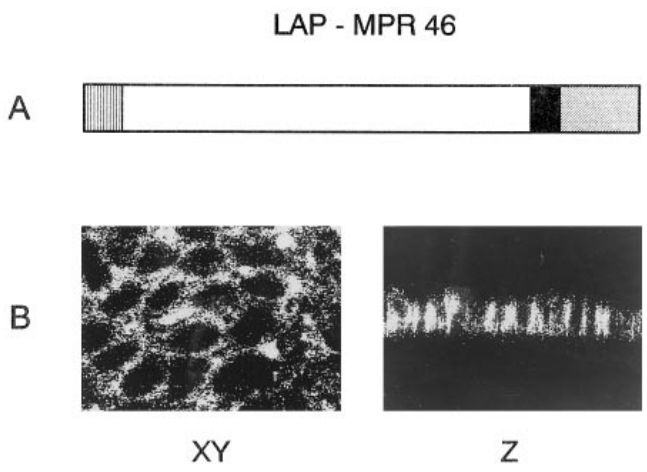

Figure 5 Cell-surface distribution of a LAP–MPR 46 chimaera in polarized MDCK cells

(**A**) Structure of the LAP–MPR 46 chimaera. The N-terminal signal sequence of LAP is indicated by a vertically hatched box. The white and the black boxes represent the luminal and transmembrane domains of LAP, and the diagonally hatched box represents the 67 residues of the cytosolic domain of MPR 46. (**B**) Filter-grown MDCK cells expressing the LAP–MPR 46 chimaera were fixed without permeabilization and exposed to a rabbit antiserum against LAP. Rhodamine-conjugated immunoglobulin was used as a second antibody. For further details see the legend to Figure 2.

When a secretory form of MPR 46 lacking the transmembrane and the cytosolic domain (sMPR 46) was expressed in polarized MDCK cells, the sMPR 46 was recovered in the apical secretions (Figure 4). The processing of the N-linked oligosaccharides of sMPR 46 to complex type oligosaccharides was comparable to that of the wild-type MPR 46 (Figure 1).

The cytosolic domain of MPR 46 was fused to the luminal and transmembrane domain of LAP. The tail-less form of LAP (LAP-RP3 in [35]) is known to be sorted to the apical domain in MDCK cells [35]. The LAP–MPR 46 chimaera was sorted to the basolateral plasma membrane domain of polarized MDCK cells (Figure 5). We conclude from these results that the cytosolic domain of MPR 46 is necessary and sufficient to ensure basolateral targeting of the receptor and a reporter polypeptide.

The 19 juxtamembrane cytosolic residues are sufficient for basolateral sorting

To define the sequences in the cytosolic domain of MPR 46 that are necessary for basolateral sorting, we analysed the surface expression of MPR 46 mutants in which the cytosolic tail was progressively truncated. The surface expression of MPR 46 mutants in which the codons for residues 12, 20, 24, 30, 50 and 61 had been converted into stop codon was analysed in polarized MDCK cells. Mutants with a cytosolic tail of 19 residues or more were expressed at the basolateral domain similarly to wild-type MPR 46, whereas a mutant with a cytosolic tail of 11 residues was apically expressed similarly to the MPR 46 stop 6 mutant (Figure 6). The apical localization of the MPR 46 stop 12 and stop 6 mutants was also evident from the binding of [^{125}I]10C6 to filter-grown MDCK cells. For the stop 12 mutant 61% of the cell-surface-binding sites were detected at the apical membrane, and for the stop 6 mutant this figure was 79% (results not shown). The expression level of the truncated receptor was generally between 10% and 50% of that of wild-type MPR 46 except for the stop 6 mutant, which had an expression level comparable to wild-type MPR 46. Saturation of a sorting mechanism can therefore be excluded as a cause for the apical sorting of the stop 12 and stop 6 mutants. We conclude from the surface distribution of the truncation mutants that the N-terminal 19 residues of the cytosolic tail are sufficient to ensure basolateral targeting.

The fraction of the truncated receptor expressed at the cell surface varied between 3.4% and 13.9% and there was no correlation with the length of the tail or the surface polarity of the mutant (Table 1). For some of the truncated MPR 46 forms (stop 20, stop 24, stop 50 and stop 61) a variable fraction of the

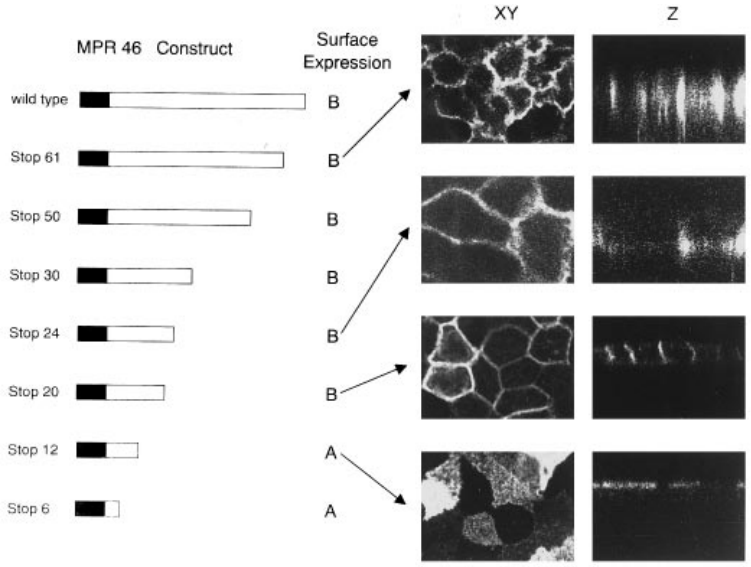

Figure 6 Cell-surface distribution of truncated forms of MPR 46 in polarized MDCK cells

Left panel: scheme depicting the C-terminal part of the transmembrane domain (black box) and the relative length of the cytosolic tail (open box) and gives the polarity of the cell-surface distribution (A, apical; B, basolateral) deduced from indirect immunofluorescence and binding of [^{125}I]-10C6. Right panel: surface distribution of truncated forms of MPR 46 in polarized MDCK cells. For details see the legend to Figure 2.

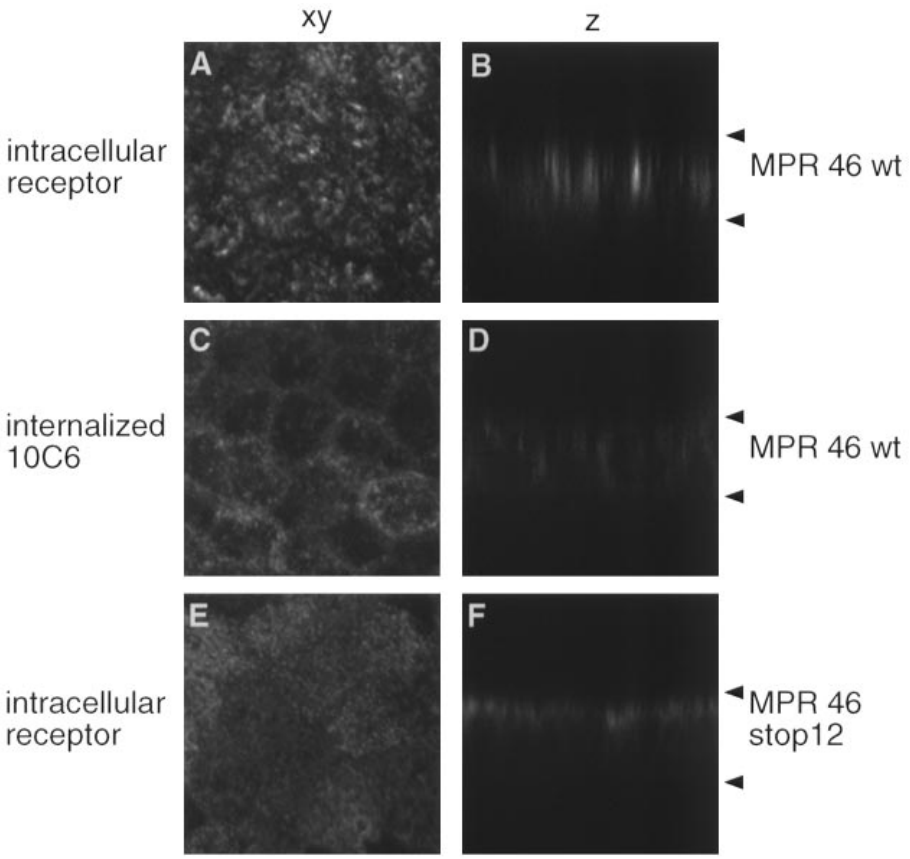

Figure 7 Distribution of MPR 46 and internalized mAb 10C6

MDCK cells expressing wild-type (wt) MPR 46 (**A–D**) or the MPR 46 stop 12 mutant (**E, F**) were grown on filters. In (**A**), (**B**), (**E**) and (**F**) the cells were fixed and exposed to mAb 10C6 after permeabilization. In (**C**) and (**D**) the cells were incubated at 37 °C for 13 min in the presence of 0.2 mg/ml mAb 10C6 in the medium of the basal chamber. After removal of the antibody and incubation for 2 min at 37 °C in medium without antibody, the cells were fixed, permeabilized and stained for mouse immunoglobulin. Immunofluorescence was analysed with a confocal laser scanning microscope. A series of optical sections from the apical to the basolateral pole of the cells was recorded. On the left the horizontal XY sections generated by an overlay of all focal planes are shown and on the right the vertical scan (Z). The apical and basal borders of the MDCK cell layer are indicated by arrowheads.

receptors contained exclusively oligosaccharides sensitive to endoglycosidase H (shown for stop 50 and stop 61 in Figure 1), suggesting that these receptor forms did not exit from the endoplasmic reticulum. When examined by indirect immunofluorescence, the bulk of the MPR 46 stop 50 and stop 61 mutants, which are represented mainly by non-processed forms, was localized in endoplasmic reticulum-like structures (results not shown), similarly to the distribution of the stop 6 mutant. Because only a fraction of the truncated receptors seem to be able to exit from the endoplasmic reticulum, the percentage of truncated receptors expressed at the cell surface would seem much higher if referred to the number of receptors that are transport competent.

The intracellular distribution of the basolaterally sorted wild-type MPR 46 in polarized cells was compared with that of the apically sorted stop 12 mutant, which shows normal processing of its oligosaccharides and is therefore thought to pass freely into the endoplasmic reticulum and Golgi. Wild-type MPR 46 is concentrated in vesicular structures, which are distributed over the entire cytoplasm as indicated by confocal sectioning (Figures 7A and 7B). mAb 10C6 internalized from the basolateral side during an incubation at 37 °C for up to 1 h (shown in Figures 7C and 7D for a 13 min incubation period) was concentrated in vesicular structures preferentially located close to the lateral membrane, producing a honeycomb pattern in horizontal sections. When the antibody was added to the apical medium, no uptake was detectable. In contrast with wild-type MPR 46, the apically sorted stop 12 mutant was concentrated in vesicular structures close to the apical surface (Figures 7E and 7F). In cells expressing the stop 12 mutant, no detectable amounts of 10C6 were internalized from the apical medium or from the basolateral medium.

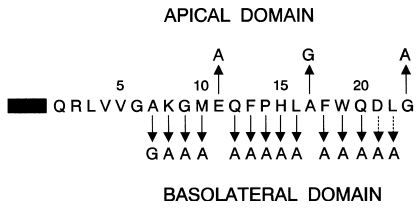

Figure 8 Surface polarity of MPR 46 stop 24 substitution mutants in MDCK cells

The amino acid sequence of the cytosolic domain of the MPR 46 stop 24 mutant is given in the single-letter code. Residues 7–23 were individually replaced by alanine or glycine residues and the sorting of the mutants to the apical or basolateral domain is indicated by the arrows. Broken arrows point to the domain to which the majority of the mutant was sorted. The expression level of the substitution mutants (mean values for binding of [^{125}I]-10C6 varied from 7373 to 25297 c.p.m. per 3 cm dish) were in the range of that of the truncated forms of MPR 46 (see Table 1).

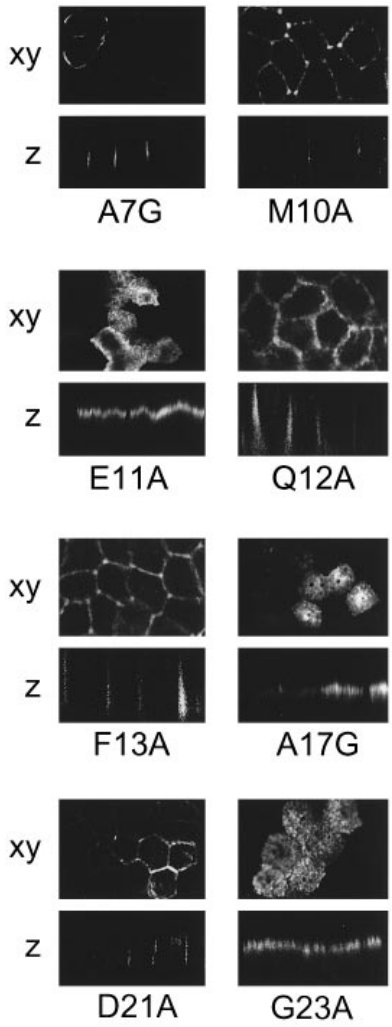

Figure 9 Cell-surface distribution of MPR 46 stop 24 substitution mutants detected by confocal microscopy

Filter-grown MDCK cells expressing the MPR 46 stop 24 mutants with substitution of the indicated residues (see also Figure 8) were fixed and exposed to mAb 10C6 from both sides. For details see the legend to Figure 2. Shown are the horizontal focal section (XY) through the middle or the apical region of the MDCK monolayer and the vertical profiles (Z).

Three separate residues are critical for basolateral sorting of MPR 46 with a cytosolic tail truncated distal to residue 23

To identify residues critical for basolateral sorting of MPR 46 in MDCK cells, residues 7–23 of the cytosolic tail of the MPR 46 stop 24 mutant were individually replaced by an alanine or a glycine residue. The point mutants were stably expressed in MDCK cells and their surface distribution was analysed by indirect immunofluorescence.

The results are summarized in Figure 8 and the confocal microscopy sections of selected mutants are shown in Figure 9. Replacement of Glu-11 or Gly-23 by alanine or of Ala-17 by glycine reversed the cell-surface polarity of the truncated receptor from basolateral to apical. Replacement of Asp-21 and Leu-22 by alanine decreased slightly the efficiency of basolateral sorting. A minor fraction of these receptor mutants became detectable by indirect immunofluorescence at the apical surface (not visible for the MPR 46 stop 24 D21A mutant in Figure 9). Replacement of the remaining residues did not affect the surface polarity.

DISCUSSION

Basolateral sorting of MPR 46

MPR 46 contains a basolateral sorting signal in its cytosolic domain that is necessary and sufficient to direct the receptor and a reporter polypeptide to the basolateral surface domain. In the absence of the cytosolic domain the receptor is transported to the apical surface domain, and if in addition the transmembrane domain is deleted, secretory forms of the receptor are delivered into the apical medium. The apical sorting of the tail-less and of the secretory form of the receptor is likely to involve the N-linked oligosaccharides attached to the luminal domain of the receptor [36].

The steady-state concentration of the receptor at the surface (less than 2 % of the total) is too small to permit the determination of whether the polarity of the receptor distribution is achieved by direct delivery of newly synthesized receptor from the TGN to the basolateral surface as generally observed in MDCK cells [5–9] or whether it is established indirectly by redistributing the receptor from the apical to the basolateral surface. Furthermore it is not clear whether basolateral polarity is established by sorting at the TGN and/or by sorting in basolateral endosomes. If basolateral sorting depends on sorting in the TGN and in basolateral endosomes, the same signal might be used at both sites. There is indirect evidence that the bulk of MPR 46 cycles between the TGN and basolateral compartments. Overexpression of MPR 46 enhances the basolateral secretion of lysosomal proteins in MDCK cells. In non-polarized cells it had been shown that this paradoxical effect of MPR 46 overexpression is likely to be dependent on the binding of newly synthesized lysosomal enzymes to MPR 46 in the TGN and the trafficking of the receptor–ligand complexes to early endosomes (or the cell surface), where the ligand dissociates and can exit into the medium [33]. The observation that overexpression of MPR 46 induces the secretion of newly synthesized lysosomal enzymes exclusively into the basolateral medium strongly suggests that MPR 46–ligand complexes formed in the TGN are delivered to a basolateral compartment. It is not clear whether receptors that are sorted to the apical plasma membrane also return to the TGN. Loss of sorting to the basolateral membrane is associated with a profound change in the intracellular distribution of the receptors. Whereas basolaterally sorted receptors are found in vesicular structures distributed throughout the cytoplasm, apically sorted receptors are concentrated in vesicular structures below the apical surface.

Sufficiency of 19 juxtamembrane cytosolic residues for basolateral sorting

Progressive truncation of the cytosolic tail revealed that the 19 juxtamembrane residues are sufficient for basolateral sorting of MPR 46. To identify residues critical for basolateral sorting, alanine/glycine-scanning mutagenesis was performed in a receptor mutant retaining the 23 juxtamembrane residues of the 67 residues of the cytosolic tail. This allowed a comparison of residues critical for basolateral sorting with those critical for rapid internalization (see below).

The only residues within the 19 juxtamembrane residues critical for basolateral sorting were Glu-11 and Ala-17. Interestingly, none of the three aromatic residues (Phe-13, Phe-18 and Trp-19) was critical for basolateral sorting. Identification of a residue by alanine/glycine-scanning mutagenesis as being critical for basolateral sorting does not permit the assignment of this residue as part of a sorting signal, because the effect might be of an indirect

nature. The basolateral signal within the 19 juxtamembrane residues might be of a conformational nature, tolerating the replacement of individual residues by alanine or glycine.

Replacement of Gly-23, a residue clearly not essential for basolateral sorting, reversed the polarity of MPR 46 distribution to the apical domain. The effect of the G23A substitution must be of an indirect nature, either by affecting the conformation of the basolateral signal within the 19 juxtamembrane residues or by impairing its accessibility. The effects of the D21A and L22A substitution, which led to a minor impairment of the sorting, can be explained in a similar manner. For the basolateral sorting signals in LAP [16] and the low-density lipoprotein receptor [14,15] it has previously been shown that the substitution of residues that are clearly located outside the sequence necessary for basolateral sorting can indirectly affect the efficiency of the sorting signals.

The basolateral sorting signal of MPR 46 can be assigned to neither the tyrosine-based nor to the dileucine-based basolateral signals. Furthermore it lacks obvious sequence similarity to the non-tyrosine/non-dileucine-based basolateral sorting signals of the transferrin receptor [19], of the polymeric immunoglobulin receptor [18] and of the low-density lipoprotein receptor [15]. The peptide corresponding to the 17 juxtamembrane cytosolic residues of the polymeric immunoglobulin receptor has in solution a propensity to adopt a β-turn, which is followed by a nascent helix structure [18]. Similar structural features have been found for the peptide corresponding to the 19-mer cytosolic tail of LAP, which contains a tyrosine-based internalization/basolateral sorting signal [37]. Two-dimensional NMR spectroscopic analysis of peptides corresponding to other non-tyrosine/non-dileucine-based basolateral sorting signals are lacking. The present study cannot exclude the possibility that the cytosolic tail of MPR 46 contains additional basolateral sorting signals C-terminal to residue 19 of the cytosolic tail, as in the cytosolic tails of the invariant chain of the MHC II complex [38,39] and of the low-density lipoprotein receptor [14], where at least two independent basolateral sorting signals have been identified.

Distinction of the basolateral sorting signal from an internalization signal located within the 23 juxtamembrane cytosolic residues

The cytosolic tail of MPR 46 contains at least three distinct internalization signals. Inactivation of one of the signals does not neccessarily impair the internalization efficiency, because of redundancy [24]. One of these internalization signals is located within the 23 juxtamembrane residues. When separated from the remaining internalization signals it sustains about one-third of the internalization rate mediated by the full-length cytosolic tail. Critical residues for internalization are Ala-7, Glu-11, Gln-12 and Phe-13 [24]. The internalization signal therefore shares with the basolateral sorting signal the dependence on Glu-11, whereas the other residues critical for internalization or basolateral sorting are clearly different (Figure 10). To what extent the two signals utilize overlapping sequences and similar structural motifs depends on the characterization of the residues establishing the binding between the sorting signals and the cytosolic proteins recognizing them.

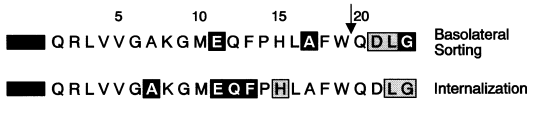

Figure 10 Comparison of residues critical for basolateral sorting or internalization within the 23 juxtamembrane cytosolic residues

Basolateral sorting or rapid internalization were abolished if residues marked by black boxes were replaced by alanine or glycine residues, whereas the replacement of residues in stippled boxes impaired basolateral sorting or internalization. The arrow indicates the C-terminal end of the juxtamembrane tail sequences required for basolateral sorting.

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