Basolateral Sorting of the Cation-dependent Mannose 6-Phosphate Receptor in Madin-Darby Canine Kidney Cells

IDENTIFICATION OF A BASOLATERAL DETERMINANT UNRELATED TO CLATHRIN-COATED PIT LOCALIZATION SIGNALS

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In polarized Madin-Darby canine kidney (MDCK) cells, sorting of membrane proteins in the trans-Golgi network for basolateral delivery depends on the presence of cytoplasmic determinants that are related or unrelated to clathrin-coated pit localization signals. Whether these signals mediate basolateral protein sorting through common or distinct pathways is unknown. The cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor (CD-MPR) contains clathrin-coated pit localization signals that are necessary for endocytosis and lysosomal enzyme targeting. In this study, we have addressed the function of these signals in polarized sorting of the CD-MPR. A chimeric protein, made of the luminal domain of the influenza virus hemagglutinin fused to the transmembrane and cytoplasmic domains of the CD-MPR was stably expressed in MDCK cells. This chimera (HCD) is able to interact with the AP-1 Golgi-specific assembly proteins and is detected on the basolateral plasma membrane of MDCK cells where it is endocytosed. Deletion analysis and site-directed mutagenesis of the cytoplasmic domain of the CD-MPR indicate that HCD chimeras devoid of clathrin-coated pit localization signals are still transported to the basolateral membrane where they accumulate. A HCD chimera containing only the transmembrane domain and the 12 membrane-proximal amino acids of the CD-MPR cytoplasmic tail is also found on the basolateral membrane but is unable to interact with the AP-1 assembly proteins. However, the overexpression of this mutant results in partial apical delivery. It is concluded, therefore, that the basolateral transport of this chimera requires a saturable sorting machinery distinct from AP-1.

The plasma membrane of polarized cells can be divided into two distinct domains, apical and basolateral, which exhibit different protein and lipid compositions. The generation and the maintenance of these domains require a continuous supply of newly synthesized components. In MDCK1 cells, newly synthesized membrane proteins destined for the basolateral or the apical surface are sorted in the trans-Golgi network (TGN) and packaged into distinct transport vesicles (1). Vesicular transport from the TGN to the apical or basolateral plasma membrane domains is mechanistically different. Although the docking/fusion of transport vesicles with the basolateral plasma membrane relies, like many transport steps, on the presence of the common fusion machinery involving NSF/SNAP proteins, the apical delivery appears to be independent of these proteins (2). More recent studies have indicated that nonpolarized cells also make use of two types of transport intermediates for the delivery of membrane proteins to the plasma membrane, one dependent on the presence of NSF/SNAP proteins and another independent of these complexes (3). Thus, polarized and nonpolarized cells have fairly similar overall organization of membrane traffic within the secretory pathway.

Thus far, two distinct features have been shown to determine sorting to the apical domain: first, the glycosylphosphatidylinositol anchor of membrane proteins (4, 5) and second, the mannose-rich core part of N-glycans present in the luminal domain of proteins (6). Many studies have now illustrated that sorting of membrane proteins to the basolateral plasma membrane is determined by the presence of specific, dominant protein determinants in their cytoplasmic domains (reviewed in Ref. 7). Extensive mutagenesis has uncovered two types of sorting motifs for basolateral delivery. First, there are those related to signals for clathrin-coated pit localization, which either rely on a key tyrosine residue, like those found in the LDL receptor (proximal determinant) (8), the vesicular stomatitis virus G protein (9), and lysosomal membrane glycoproteins (10), or on a di-leucine motif, like in the IgG Fc receptor (11). Second, there are basolateral targeting signals that are unrelated to determinants for clathrin-coated pit localization. Examples can be found in the LDL receptor (distal determinant) and in the poly-IgA receptor (8, 12, 13). Interestingly, the same (or a very closely related) basolateral sorting signal can mediate the recycling of endocytosed membrane proteins from endosomes back to the plasma membrane (14, 15). The similarities between the determinants responsible for endocytosis, basolateral sorting, and plasma membrane recycling suggest that these processes are extremely related and involve similar sorting machineries that remain to be characterized.

In addition to sorting membrane proteins destined for the apical or basolateral domains in the TGN, the polarized MDCK cells must also sort their newly synthesized lysosomal hydro-
lases bound to the mannose 6-phosphate receptors (MPRs). Previous studies have shown that one of the two MPRs, the mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGF II), traffics within the basolateral domain of MDCK cells because it can be detected on the basolateral membrane of these cells (16). In nonpolarized cells, the endocytosis of this receptor relies on a tyrosine-based motif, whereas that of the other MPR, the cation-dependent mannose 6-phosphate receptor (CD-MPR), requires a weak tyrosine-based motif and a dominant motif containing two phenylalanine residues (17). On the other hand, efficient lysosomal enzymatic targeting requires the presence of a di-leucine-based motif present at the carboxyl terminus of both MPRs (18–20). In addition, the signals required for efficient endocytosis of the MPRs contribute, although weakly, to efficient lysosomal enzyme targeting (19). The MPRs and their bound lysosomal enzymes are known to be sorted in the TGN via clathrin-coated vesicles. The first step in the formation of these transport intermediates is the interaction of the AP-1 Golgi-specific assembly proteins with TGN membranes. The MPRs are part of the membrane components that permit the efficient recruitment of AP-1 on membranes (21–23), a process regulated by the small GTPase Arf-1 (24). In the case of the CD-MPR, specific determinants in its cytoplasmic domain, in particular a casein kinase II phosphorylation site are required for high affinity interaction of AP-1 with TGN membranes (25).

In this study, we have investigated the sorting of the CD-MPR in polarized MDCK cells. For this, we have stably expressed a chimeric protein made of the luminal domain of the influenza virus hemagglutinin (HA) fused to the transmembrane and cytoplasmic tail of the CD-MPR. This HCD chimeric protein traffics within the basolateral domain and can be detected at the basolateral surface. Mutations of the different sorting signals proposed to mediate the interaction of the CD-MPR tail either with the Golgi-specific assembly proteins AP-1 or its plasma membrane counterpart AP-2 do not affect the basolateral delivery of the corresponding HCD chimeras. Truncations of the cytoplasmic domain indicated that a sorting determinant, unrelated to motifs necessary for clathrin-coated pit localization, is present in the membrane-proximal part of the CD-MPR cytoplasmic domain or the transmembrane domain, which confers basolateral targeting. This determinant, neither supports the AP-2-dependent endocytosis nor triggers the recruitment of AP-1 on membranes. This strongly suggests that an additional sorting machinery that recognizes signals unrelated to those mediating clathrin-coated pit localization could be responsible for basolateral targeting of membrane proteins in MDCK cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells (strain II) were grown as described (26). PA317 amphotropic retrovirus packaging cells (27) were maintained in MEM supplemented with 10% fetal calf serum, 4 mM glutamine, and PA317 amphotropic retrovirus packaging cells (27) were maintained in MEM supplemented with 10% fetal calf serum, 4 mM glutamine, and 0.5% BSA (MEM-BSA). Cells were seeded with 0.5 to 1 x 10^6 cells each, and cell monolayers were used for experiments 4 days after plating.

Basolateral Sorting of the CD-MPR in MDCK Cells

HCD-A1 (pBD28) and HCD-A13A18A45 (pBD19) were generated by ligation of two complementary oligonucleotides with the sequences 5'-GATGATGGG-3' and 5'-GATCCCCATCAT-3' between the BamHI and HindIII sites of HCD, introducing a stop codon at Tyr45 in the cytoplasmic domain. The chimeric gene HCD-A13A18A45 (pBD25) was generated by ligation of two complementary oligonucleotides with the sequences 5'-ATGAGCAGTGGG-3' and 5'-GATCCTCAGTCGTCATTCCC-3'. This double-stranded DNA fragment was inserted between the NotI and BamHI sites of HCD and HCD-A1, respectively, creating a stop codon at residue 13 (Phe) of the cytoplasmic domain. Stably transfected MDCK cells were used to infect MDCK cells. Stably transfected MDCK cells were selected in medium containing 0.8 mg/ml G418 (Life Technologies, Inc.) and cloned using glass cylinders. Cells expressing the protein of interest were identified by immunofluorescence. At least two independent clones expressing the highest levels of the respective chimeric protein were used for further experiments. All transfected cell lines were used fully polarized as judged by methionine uptake (basal/ apical ratio greater than 4:1) (33) and secretion of an endogenous glycoprotein complex (34). Prior to experiments the tightness of monolayers was assessed with [3H]inulin (Amersham Corp.) (35). All experiments were performed using cell lines of passage numbers 5 through 10 after cloning.

Cell Surface Transport Assay—MDCK cells grown on Costar Transwell units were rinsed with warm PBS+ (PBS containing 0.9 mM CaCl2 and 0.5 mM MgCl2) and starved for 60 min in MEM lacking methionine and methionine (Select Amine-kit; Life Technologies, Inc.) containing 0.35 g/liter sodium bicarbonate, 20 mM Hepes, pH 7.3, and 0.5% BSA (MEM-BSA). Cells were then pulse labeled in a wet chamber containing 0.35 g/ml Expre35S (1000 Ci/mmol, 10 mCi/ml) NEN Life Science Products). One set of filters was washed three times with cold PBS+ and placed on ice in MEM-BSA; the other sets of filters were chased at 37 °C in MEM-BSA with a 100-fold excess methionine and cysteine. At the end of the chase cells were washed with cold PBS++ and placed on ice in MEM-BSA. Subsequent steps were performed at 4 °C. Cells were washed once with MEM-BSA and incubated either from the apical or basolateral side with a 1:500 dilution of an hemagglutinin antiserum (monoclonal antibody H269, generous gift of J. Shkei) in MEM-BSA for 90 min on a rocking platform. In some experiments the antibody was included in the apical or basolateral chase medium and allowed to bind for another 90 min on ice. The excess unbound antibodies were removed over 30 min by three washes with MEM-BSA and one wash with PBS++, filters were cut out of the holder, and cells were lysed in the presence of an excess of unlabeled protein.

For preparation of unlabeled lysates a cell line overexpressing the wild type HA was grown to confluency on 10-cm culture dishes. Cells from one dish were lysed on ice in 2.5 ml of B1 (50 mM Tris, pH 7.2, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS containing freshly added protease inhibitor mixture (2 mg/ml leupeptin, 2 mg/ml aproti- nin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride)). The lysate was cleared by centrifugation for 5 min at 12,000 x g, and 1 ml of supernatant was used to lyse labeled cells. The lysate was centrifuged in an Eppendorf microfuge for 5 min to remove debris. An aliquot (one-tenth of the lysate) was supplemented with antibody (anti-HA, mAb H269) and incubated overnight at 4 °C, and total labeled protein was isolated by the addition of protein A-Sepharose. The second aliquot (nine-tenths of the lysate) was frozen in liquid nitrogen and stored overnight at −70 °C. Labeled protein that had appeared on the cell surface and thus had bound antibody was precipitated by the addition of protein A-Sepharose. Precipitates were washed three times.
with B1, twice with B2 (50 mM Tris, pH 7.2, 100 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 0.5% SDS, 0.5% deoxycholate), twice with B3 (50 mM Tris, pH 7.2, 500 mM NaCl, 2 mM EDTA, 0.1% Triton X-100), and once with B4 (50 mM Tris, pH 7.2, 100 mM NaCl, 2 mM EDTA). Finally, proteins were released from the beads by boiling in Laemmli sample buffer and analyzed by SDS-PAGE on a 10% polyacrylamide gel (36). The band intensities were calculated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the amount of protein transported to the cell surface was expressed as the percentage of the total immunoprecipitated protein. In some experiments cells were pretreated with 10 mM butyric acid (Sigma) 12 h prior to labeling to induce transcription of stably transfected cDNA constructs (8).

**Sucrose Velocity Gradient Centrifugation—**MDCK cells grown on 60-mm plastic dishes to subconfluence and treated with 10 mM NH₄Cl 12–16 h prior to the experiment to reduce the endogenous level of lysosomal proteases were metabolically labeled essentially as described above except that cells were labeled for 1 h and chased for 3 h. After labeling cells were lysed, and an aliquot of the lysate was analyzed by sucrose gradient centrifugation as described (37). Gradient fractions were immunoprecipitated with anti-HA and analyzed by SDS-PAGE and fluorography.

**Internalization Assay Based on Surface Biotinylation—**Internalization rates of CD-MPR chimeras were determined by the surface biotinylation assay described in Ref. 38, except that MESNa was used for stripping the cell surface biotin. Biotinylated CD-MPR chimeras were detected by Western blotting with 125I-labeled streptavidin. Signals were quantified using a PhosphorImager.

**AP-1 Recruitment—**HeLa cells were grown on coverslips in α-MEM supplemented with 10% fetal calf serum, 10 mM Hepes, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were washed in medium devoid of serum and then incubated for 30 min with a recombinant virus that expresses the T7 polymerase gene (39). After washing the cells in medium supplemented with 5 mM hydroxyurea, the N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammoniummethylsulfate reagent (Boehringer Mannheim) was used to transfect the cells with the indicated constructs (HCD and HCD-Δ55) cloned in pGEM1 vector, following the manufacturer’s instructions. After 1 h, the cells were washed and allowed to express for 2–3 h in medium supplemented with 5 mM hydroxyurea. Pulse-chase experiments performed in parallel indicated that similar levels of HCD and HCD-Δ55 were expressed under those conditions and transported through the secretory pathway with similar kinetics (data not shown). Cells were then fixed on MDCK cells expressing either the wild type HA or the HCD and immunoprecipitated these labeled proteins using a monoclonal antibody directed against the luminal domain of HA. After a 20-min pulse, the HA and the HCD were found in low molecular weight, immature forms that were rapidly converted upon a chase into higher molecular weight, mature forms reflecting the conversion of high mannose to complex type oligosaccharides (Fig. 2). These results indicate that the HCD chimera moves efficiently through the secretory pathway with similar rates as the HA (t½ ~ 30 and 25 min, respectively). Such pulse-chase experiments were also performed with the HCD-Δ55, which harbors a 55-amino acid-long truncation of the introduced amino acid and the position of the replacement.

**RESULTS**

In MDCK cells, the Man-6-P/IGF II receptor traffics between the TGN and the endocytic organelles of the basolateral domain and is found on the basolateral plasma membrane (16). To study the transport of the CD-MPR in these cells, we have generated MDCK clones stably expressing chimeric proteins made of the luminal domain of the influenza virus HA, an apically sorted membrane glycoprotein, fused to the transmembrane and cytoplasmic domain of this receptor (Fig. 1) and performed pulse-chase experiments followed by cell surface immunoprecipitations to measure the appearance of these chimeric proteins on the apical or the basolateral domain.

**HA/CD-MPR Chimeras Are Normally Transported through the Secretory Pathway and Are Present on the Basolateral Membrane of MDCK Cells—**The normal transport of HA to the cell surface depends on its proper folding and trimerization (37). Because modifications in the transmembrane and cytoplasmic domains of HA can affect its normal rate of transport (40, 41) we determined whether the HA/CD-MPR chimera (HCD construct) is properly folded and transported through the secretory pathway. We first performed classical pulse-chase experiments on MDCK cells expressing either the wild type HA or the HCD and immunoprecipitated these labeled proteins using a monoclonal antibody directed against the luminal domain of HA. After a 20-min pulse, the HA and the HCD were found in low molecular weight, immature forms that were rapidly converted upon a chase into higher molecular weight, mature forms reflecting the conversion of high mannose to complex type oligosaccharides (Fig. 2). These results indicate that the HCD chimera moves efficiently through the secretory pathway with similar rates as the HA (t½ ~ 30 and 25 min, respectively). Such pulse-chase experiments were also performed with the HCD-Δ55, which harbors a 55-amino acid-long truncation of
the CD-MPR tail (Fig. 2). The rate of transport of this mutant was slightly slower than that of HCD ($t_{1/2}$ = 45 min), indicating that the deletion of the CD-MPR tail only moderately affects the transport of the corresponding chimera. The other mutants used in this study were transported through the secretory pathway with similar rates as that of HCD− used in this study were transported through the secretory pathway. The transport of the corresponding chimera. The other mutants that the deletion of the CD-MPR tail only moderately affects the rate of transport of this mutant was slightly slower than that of HCD ($t_{1/2}$). The rate of transport of this mutant was slightly slower than that of HCD ($t_{1/2}$). The rate of transport of this mutant was slightly slower than that of HCD ($t_{1/2}$).

**Fig. 2. Intracellular transport of wild type HA and HA/CD-MPR chimeras in MDCK cells.** Transfected cells were pulse labeled with $[^{35}S]$methionine/cysteine for 20 min and chased for various periods of time as indicated. Cells were solubilized with Triton X-100 and HA, and the HA/CD-MPR chimeras were immunoprecipitated with the anti-HA monoclonal antibody, resolved by SDS-PAGE, and visualized by fluorography as described previously (25). Duplicates are shown for each time point. Fluorographs were quantified by PhosphorImager. The kinetics of maturation of the HA and the HA/CD-MPR chimeras is represented by the disappearance of the precursor (immature) form based on cell surface biotinylation, the endocytosis rate of HCD was found to be $\sim$2.6%/min (Table I). Because only minor amounts of HCD could be detected at any time on the apical surface even when the antibody was present during the chase, we conclude that HCD is vectorially transported from the TGN for trafficking within the basolateral domain. These results suggest that either the transmembrane or the cytoplasmic domain of the CD-MPR contains a dominant basolateral sorting determinant.

**Fig. 3. Oligomerization of wild type HA and HA/CD-chimeras.** Transfected cells were either pulse labeled with $[^{35}S]$methionine/cysteine for 1 h ($P$) or pulse labeled for 1 h followed by a 3-h chase ($C$). Cell lysates were centrifuged on 5–25% (w/v) sucrose gradients containing 0.1% Triton X-100. Each fraction was immunoprecipitated with anti-HA and analyzed by SDS-PAGE and fluorography.

**Basolateral Sorting of the CD-MPR in MDCK Cells**

Mutations of Endocytosis Sorting Signals Do Not Affect Basolateral Transport of HA/CD-MPR Chimeras—To decipher the determinants important for sorting to the basolateral domain, we introduced several deletions and point mutations in the CD-MPR cytoplasmic tail (Fig. 1). The mutations were designed to affect the sequences known to be important for intracellular trafficking of the CD-MPR. We first deleted the carboxyl-terminal di-leucine motif alone (construct HCD−Δ55) or together with the adjacent casein kinase II phosphorylation site (HCD−Δ17), shown to be critical for efficient lysosomal enzyme targeting (18, 25). Table I shows that after a 40-min chase, 80% of these mutant proteins present on the cell surface were still detected on the basolateral plasma membrane. Pulse-chase experiments followed by cell surface immunoprecipitation indicated that the mutant proteins were vectorially transported from the Golgi complex to the basolateral domain (data not shown). The endocytosis of the CD-MPR is mediated by two independent endocytosis motifs, a dominant determinant containing phenylalanine 13 and 18 and a weak determinant containing the tyrosine 45 (17). We therefore replaced these three critical amino acids in the HA/CD-MPR chimera by alanine residues (construct HCD−A13A18A45). Indeed, these mutations significantly reduced the endocytosis rate of the chimera by 60% (Table I). However, the sorting of this mutant was not affected, and 90% of the cell surface protein was still found on the cell surface even when the antibody was present during the chase.
the basolateral plasma membrane. Therefore, it appears that neither the endocytosis motifs nor the sequence important for lysosomal enzyme delivery are essential for basolateral transport when mutated individually.

We introduced further truncations in the CD-MPR cytoplasmic domain to remove both the tyrosine 45 and the phenylalanine 13 and 18 (construct HCD-Δ55). As shown in Table I, this mutant protein was mostly targeted to the basolateral domain (more than 80%). Together, these results indicate that an additional signal(s), unrelated to those mediating clathrin-coated pit localization, must be present to mediate basolateral transport of the most truncated form of the HA/CD-MPR chimera.

**Basolateral Sorting of HCD-Δ55 Is Saturable**—Signal-mediated sorting of membrane proteins in the secretory pathway of MDCK cells can be saturated by overexpressing these membrane proteins. This has been observed for the newly synthesized Ig 120 (10) or LDL receptor (8). To test the possibility that basolateral sorting of the most truncated HCD construct was signal-mediated and could become saturated, we selected MDCK clones highly expressing the HCD-Δ55, A-1 construct (~7-fold overexpression compared with HCD) and performed pulse-chase experiments followed by cell surface immunoprecipitation. Fig. 5 shows that high levels of expression of this

![Cell surface delivery of wild type HA and the HCD in MDCK cells](image)

**FIG. 4.** Cell surface delivery of wild type HA and the HCD in MDCK cells.

Filter grown MDCK cells expressing wild type HA or the HCD were pulse labeled with [35S]methionine/cysteine for 20 min and subsequently chased for 0–80 min. At each time point anti-HA was added to the apical (A) or basolateral (B) medium and allowed to bind on ice. Cells were lysed, and one-tenth of the sample was removed to determine the total amount of labeled HA or HCD. The rest of the lysate was incubated with protein A-Sepharose beads, and bound proteins were analyzed by SDS-PAGE and fluorography. Fluorographs were quantified by PhosphorImager, and the values for recovered cell surface protein were expressed as the percentage of total mature protein.

**TABLE I**

Polarized surface expression and internalization of HA/CD-MPR chimeras

| Protein   | Level of expression | Polarized surface expression | Surface-to-intracellular distribution | Number of experiments | Internalization rate |
|-----------|---------------------|------------------------------|---------------------------------------|-----------------------|---------------------|
|           |                     | Apical % | Basolateral % | % surface |                     | %/min              |
| HA        | 2.0                 | 76 ± 7  | 24 ± 7        | 33 ± 14    | 3                    | <0.1 (n = 3)       |
| HCD       | 1.0                 | 11 ± 3  | 89 ± 3        | 1.7 ± 0.8  | 3                    | 2.6 (n = 2)        |
| HCD-Δ5    | 0.7                 | 20 ± 6  | 80 ± 6        | 1.6 ± 0.4  | 3                    | ND                 |
| HCD-Δ17   | 0.5                 | 20 ± 6  | 80 ± 4        | 3.7 ± 0.7  | 3                    | ND                 |
| HCD-Δ23   | 1.2                 | 13 ± 9  | 87 ± 9        | 10 ± 5     | 5                    | ND                 |
| HCD-Δ55   | 0.6                 | 13 ± 8  | 87 ± 8        | 10 ± 4     | 8                    | 0.3 (n = 3)        |
| HCD-Δ55,A-1| 0.4               | 15 ± 5  | 82 ± 5        | 11 ± 6     | 4                    | ND                 |
| HCD-Δ17,A13A18A45 | 0.6 | 9 ± 9  | 91 ± 1        | 12 ± 5     | 4                    | 0.9 (n = 5)        |
| HCD-Δ17,A13A18A45 | 0.6 | 8 ± 4  | 92 ± 4        | 9 ± 3      | 2                    | 0.2 (n = 2)        |

*The expression levels of the chimeras were determined by a 20-min pulse with [35S]methionine followed by immunoprecipitation as described under “Experimental Procedures.” These values were normalized using the endogenous cation-independent MPR as a reference. The expression level of HCD was about 3-fold higher than that of cation-independent MPR and was arbitrarily set to one. The polarized surface expression of the HA and HCD chimeras was determined after a 20-min pulse with [35S]methionine and a 40-min chase using cell surface immunoprecipitation. Values are given as the percentage of total cell surface protein and represent the mean values ± S.E. The values between brackets were obtained in experiments where the anti-HA antibody was present in the medium during the chase. The values are expressed as percentages of mature protein at the cell surface after a 40-min chase. The internalization rates were calculated from the 5- and 10-min time points of the internalization assay based on cell surface biotinylation (see “Experimental Procedures”) and are expressed as the percentage (mean ± S.E.) of surface-biotinylated protein that acquired resistance to MesNa. ND, not determined.
mutant protein resulted in a significant mis-sorting to the apical domain when compared with lower expression levels. Thus, there is a clear correlation between overexpression of this mutant and apical delivery. The appearance on both plasma membrane domains occurred at similar initial rates, strongly suggesting that the mis-sorting of this truncation mutant occurred in the trans-Golgi network. These results suggest that the 12-amino acid-long sequence adjacent to the CD-MPR transmembrane domain and/or the CD-MPR transmembrane domain contains a determinant for basolateral transport and that the corresponding sorting machinery could become saturated upon overexpression of this chimera.

**DISCUSSION**

We have expressed in the MDCK cells chimeric proteins made of the transmembrane and cytoplasmic domain of the CD-MPR fused to the luminal domain of the apically directed hemagglutinin to study CD-MPR trafficking in polarized cells. We show that 1) the chimeric protein traffics within the basolateral domain, 2) the mutagenesis of determinants related to clathrin-coated pit localization signals in the CD-MPR tail does not affect its appearance on the basolateral membrane, 3) an additional sorting determinant is present in the cytoplasmic domain and/or the transmembrane domain of CD-MPR that mediates basolateral transport, and 4) this basolateral sorting determinant is not efficiently recognized by the Golgi-specific AP-1 assembly proteins and therefore may involve a different sorting machinery.

Our previous work in nonpolarized cells has shown that HA/MKP chimeras colocalize with the endogenous MPRs, indicating that the information contained within these chimeric proteins is sufficient to specify their intracellular localization (29). We have now expressed these HA/CD-MPR chimeras in polarized MDCK cells. Cell surface immunoprecipitation experiments revealed that more than 90% of the cell surface chimera...
Basolateral Sorting of the CD-MPR in MDCK Cells

**β-amyloid**

\[
\text{KKKTSIHHGVVEV...}
\]

**TGN38**

\[
\text{KASD...RLNLKL*}
\]

**CD-MPR (A55)**

\[
\text{FLQRLVGA...KMEQ*}
\]

**polyIgR**

\[
\text{RARHRE...DRVSGSYT*}
\]

**CT-MPR**

\[
\text{KKRE...VINKL7SCCRR}
\]

Fig. 8. Sequence comparison of the cytoplasmic domains of different basolaterally targeted membrane proteins. Sequences are shown in single-letter code. Triangles mark the first residue of the cytoplasmic domains, and asterisks mark the last residue. The cytoplasmic domain of the most truncated HCD chimera (HCD-A55) is aligned with the cytoplasmic tails of the β-amyloid precursor and TGN 38 based on its YQRL motif and with the polyR and cation-independent MPR cytoplasmic tails based on the RXXY motif. Tyrosine residues in the clathrin-coated pit localization motifs are highlighted with *ovals*, and the arginine and valine residues in the RXXY motifs are highlighted with *squares*.

Therefore, it appears that this membrane protein devoid of clathrin-coated pit localization signals is transported to the basolateral domain of MDCK cells via an AP-1-independent pathway.

Basolateral sorting of membrane proteins generally relies on signals located in their cytoplasmic domains. We therefore examined the 12 amino acids left in the CD-MPR cytoplasmic tail of HCD-A55 for the presence of putative sorting signals (Fig. 8). First, a YQRL motif is present that is identical to that found in TGN 38. This determinant was shown to be important for the retrieval of TGN 38 from the plasma membrane to the TGN in nonpolarized cells (45, 46) and basolateral sorting in MDCK cells (47). Hydrophobicity plots of the CD-MPR predict that the tyrosine residue in this YQRL motif is the last amino acid of the transmembrane domain (17) and that this motif would be barely accessible to the sorting machinery. However, it has been noticed that Golgi-resident proteins have on average shorter transmembrane domains (~15 residues) than plasma membrane proteins (~20 residues) (48, 49). Because the CD-MPR transmembrane domain is 20–25 amino acids long, it remained possible that in the context of the Golgi membrane the tyrosine contained in this YQRL motif could be accessible to the basolateral sorting machinery. A 15-amino acid-long transmembrane domain could provide the minimal spacing for accessibility of this tyrosine-based motif. For example, the basolateral sorting of the β-amyloid precursor is dependent upon a key tyrosine residue in the cytoplasmic tail of the protein located only 5 residues away from the transmembrane domain (50). However, mutation of the tyrosine residue to an alanine in our most truncated HCD chimera (HCD-A55-A1) did not influence its basolateral transport at low levels of expression. Therefore, this tyrosine-based motif does not appear to be essential for basolateral transport of the most truncated mutant.

A basolateral sorting determinant could reside in the RLVV sequence overlapping the YQRL motif (Fig. 8). A similar motif (HRRNV), unrelated to classical coated pit localization signals and closely located near the transmembrane domain, has been shown to mediate basolateral delivery of a truncated version of the poly-Ig receptor (13). Within this sequence motif the histidine, the first arginine, and the carbonyl-terminal valine are most essential for basolateral sorting. Two of these essential residues (arginine and valine) are conserved in the CD-MPR membrane-proximal determinant. Interestingly, the Man-6-P/-IGF II receptor cytoplasmic domain also contains a similar sequence motif (RETVE) close to the transmembrane segment (Fig. 8). Because further truncations of the 12-residue-long tail of the HCD chimera resulted in retention of the protein in the

**Fig. 7.** Quantitation of the amount of γ-adaptin associated with the TGN in cells overexpressing the wild type or mutant version of the HCD chimeras. HeLa cells were transiently transfected as in Fig. 6 and similarly processed for double indirect immunofluorescence. The intensity of the fluorescence signal corresponding to γ-adaptin (Texas Red channel) was quantitated for 122 cells (nontransfected cells), 88 cells (HCD), and 73 cells (HCD-A55) cells, and the results were processed as described under “Experimental Procedures.” Values correspond to the means ± S.E. of four independent experiments (expression of the chimera for 2 or 3 h). Using the Student’s t test, the confidence limits of the sample populations were found to be higher than 99% for HCD and not significantly different for HCD-A55 when compared with nontransfected cells.

is present at the basolateral plasma membrane indicating that this protein traffics within the basolateral domain of MDCK cells. This result agrees with that of Breuer and co-workers (44), who showed that the CD-MPR is predominantly present on the basolateral plasma membrane of MDCK cells. Thus, both the CD-MPR and the Man-6-P/IGF II receptor (16) traffic within the same cellular domain of MDCK cells. This HCD chimera is able to trigger the recruitment of the AP-1, Golgi-specific assembly proteins on membranes. Therefore, it is likely that its appearance on the basolateral membrane reflects its efficient sorting to endosomes along the AP-1-dependent pathway followed by the recycling of a fraction of this chimeric protein back to the basolateral plasma membrane.

To identify the minimal determinant required for this basolateral sorting, we first mutated the signals in the CD-MPR cytoplasmic domain known to be important for its endocytosis (phenylalanine 13 and 18 and tyrosine 45), efficient lysosomal enzyme targeting (carboxyl-terminal di-leucine motif), and high affinity binding of AP-1 on membranes (the casein kinase II phosphorylation site adjacent to the carboxyl-terminal di-leucine motif). The re-expression of CD-MPR mutants in MPR-negative fibroblasts has indicated that the mutation of either the carboxyl-terminal di-leucine motif alone or the endocytosis motif alone (phenylalanine 13 and 15 and tyrosine 45) does not affect the efficient AP-1 recruitment (25). Consistent with this notion, the same mutations introduced in the HCD chimera do not affect its basolateral delivery. This suggests that the protein is still able to follow the AP-1-dependent pathway in MDCK cells. The most striking finding is that mutations in the CD-MPR cytoplasmic domain retaining the different clathrin-coated pit localization signals do not alter the basolateral delivery of the corresponding HCD mutant (HCD-A55). Even the largest truncation of the CD-MPR tail did not result in the apical delivery of the truncation mutant (HCD-A55). Such an HCD mutant cannot trigger the recruitment of AP-1 on Golgi membranes nor can it be endocy-
endoplasmic reticulum (data not shown), we cannot exclude the remote possibility that the transmembrane domain of the CD-MPR contributes to basolateral delivery. A more likely explanation, however, is that these 12 amino acids contain a determinant (possibly the RLVV motif) that mediates basolateral delivery of HCD in the absence of the known clathrin-coated pit localization signals. The final characterization of this signal awaits more extensive mutational analysis. If this sorting determinant mediates the basolateral delivery of the luminal domain of HA used as a reporter, it also remains to be determined to what extent it contributes to CD-MPR trafficking.

It is interesting to note that the overexpression of the most truncated HCD-Δ55 mutant results in the significant delivery of the protein to the apical membrane. This observation was not only made with high expressing MDCK clones (this study) but also with low expressing MDCK clones treated with butyrate, which induces a 5–10-fold increase in the expression level (data not shown). HCD-Δ55 or HCD-Δ55-A1 were significantly missorted to the apical plasma membrane after butyrate-induced overexpression. Under such conditions, 40% of the cell surface proteins were present on the apical membrane, strongly suggesting that a saturable, AP-1-independent transport machinery recognizes a basolateral sorting determinant present in the 12-amino acid-long cytoplasmic tail and/or transmembrane domain of CD-MPR. A similar result was also obtained with the HCD-A17, A13A18A45 mutant. In contrast, such an increase in the expression level of HCD, which follows an AP-1-dependent pathway at the exit of the TGN, had almost no effect on its basolateral delivery. Thus, these two sorting machineries can be saturated in a different manner. Little is known about the machineries responsible for basolateral delivery of membrane proteins in polarized cells. Similarities between several basolateral and clathrin-coated pit localization signals have suggested that basolateral transport of membrane proteins requires coat components related to the assembly proteins of clathrin-coated vesicles. Thus far, the μ subunits of the AP-1 and AP-2 assembly proteins have been shown to interact with tyrosine-based motifs in the yeast two hybrid system (51, 52). In a similar manner, the μ subunit of the newly described AP-3 complex that shares structural similarities with AP-1 and AP-2 also recognizes tyrosine-based sorting signals (52). Several basolateral targeted membrane proteins have been shown to contain tyrosine-independent sorting determinants. It remains to be determined whether these tyrosine-independent sorting determinants can be recognized by coat components related or unrelated to the AP-1 and AP-2 assembly proteins.

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