Membrane Cofactor Protein (CD46) Is a Basolateral Protein That Is Not Endocytosed

**IMPORTANCE OF THE TETRAPEPTIDE FTSL AT THE CARBOXYL TERMINUS**

(Received for publication, April 30, 1997)

Andrea Maisner‡, Gert Zimmer‡, M. Kathryn Liszewski§, Douglas M. Lublin¶, John P. Atkinson§, and Georg Herrler‡

From the ‡Institut für Virologie, Philipps-Universität Marburg, D-35037 Marburg, Germany, the ¶Division of Rheumatology, Department of Internal Medicine, and the §Division of Laboratory Medicine, Department of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Membrane cofactor protein (MCP) is a widely distributed complement regulatory protein that is expressed on the basolateral surface of polarized epithelial cells. The basolateral targeting of the BC1 isoform of MCP was analyzed by generating deletion mutants and point mutants within the cytoplasmic tail of 16 amino acids. A sequence of four amino acids, FTSL, was found to be dispensable for the basolateral transport of MCP. This tetrapeptide has two unique features compared with the targeting motifs of other basolateral proteins: (i) it contains a phenylalanine rather than a tyrosine at position 1; (ii) it is located at the very COOH-terminal end. Replacement of the phenylalanine or the leucine by an alanine resulted in a nonpolarized delivery to the cell surface. On the other hand, substitution of a tyrosine for alanine resulted in a nonpolarized delivery to the cell surface. Therefore, the wild type protein was not subject to endocytosis. Our results indicate that the targeting signal YXX-Large aliphatic is that involved in various sorting events has been modulated in MCP in such a way that it allows basolateral transport but not endocytosis.

Polarized epithelial cells can be divided into morphological and functional subdivisions. Cellular compartmentalization and specific directional transport of cellular components are responsible for the creation of an apical and a basolateral plasma membrane domain that are separated by junctional complexes (for review, see Ref. 1). One of the best characterized epithelial cell lines is the Madin-Darby canine kidney cell line in which newly synthesized apical and basolateral proteins are sorted at the trans-Golgi network by segregation into different vesicles for direct transport to their respective membrane domain (2–5). In other polarized cells, e.g. hepatocytes, apical proteins are first delivered to the basolateral side where they are sorted and transcytosed to the apical membrane (6). In recent years, a correlation between the cytoplasmic tail of a protein and the basolateral targeting has been established (for review, see Ref. 7). For some basolateral sorting events signals involving a critical tyrosine residue, a close relationship to determinants for coated pit localization has been described (8–10). However, some other proteins, e.g. the polymeric immunoglobulin receptor or the low density lipoprotein receptor, possess unrelated targeting and endocytosis signals (11, 12). In contrast to the basolateral sorting, the apical targeting is not well characterized. For apically secreted proteins the importance of N-glycans has been demonstrated (13). However, for membrane-bound apical proteins the involvement of the carbohydrate moiety is still speculative. Proteins anchored by glycosphosphatidylinositol (GPI)1 are normally delivered to the apical plasma membrane (14, 15). Recently, it has been shown that the distribution of GPI is nonpolarized, indicating that the sorting machinery for nonprotein-linked GPs and for GPI-anchored proteins is different (16).

Membrane cofactor protein (MCP; CD46) is a type I membrane protein and is expressed on all nucleated human cells tested. MCP functions as a cofactor for the plasma serine protease factor I by binding to complement factors C3b and C4b deposited on self tissue (17). By promoting the proteolytic degradation of these factors, it protects the cell from complement-mediated damage. In addition, MCP serves as a receptor for measles virus (18, 19). For virus binding, the N-glycans of MCP are of critical importance (20–22). The extracellular portion of MCP consists of four cysteine-rich short consensus repeats (SCRs), three of which contain sites for N-glycosylation. The SCRs are followed by a serine, threonine, and proline-rich region (STP), the site of O-glycosylation. By alternative splicing multiple MCP isoforms arise which contain different combinations of the STP regions A, B, and C. A commonly expressed isoform contains the BC regions consisting of 29 amino acids. The extracellular portion of MCP is connected to the hydrophobic transmembrane domain by a short region of unknown function. The intracellular domain of the protein is divided into two parts. The membrane proximal portion, designated as the intracytoplasmic anchor, is present in all MCP isoforms and consists of 10 mainly basic amino acids. The carboxyl-terminal end of MCP, designated as cytoplasmic tail 1 or 2, is subjected to alternative splicing like the STP region. In the various isoforms of MCP either of two different tails was found: one comprising 16 amino acids (tail 1) and the other consisting of 23 amino acids (tail 2). The nomenclature of MCP isoforms indicates which segments (A, B, or C) of the STP region and which

---

1 The abbreviations used are: GPI, glycosphosphatidylinositol; MCP, membrane cofactor protein; SCR, short consensus repeat; STP, serine, threonine, proline-rich region; MDCK, Madin-Darby canine kidney; DAF, decay accelerating factor; TM, transmembrane; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; VCNA, Vibrio cholerae sialidase; wt, wild type; SA, sialic acid(s).
cytoplasmic tail (1 or 2) are expressed (for review, see Ref. 17). In a recent paper, we reported that two MCP isoforms with different tails (MCP-BC1 and MCP-BC2) were directed to the basolateral surface of polarized epithelial cells. A mutant lacking the cytoplasmic tail was transported in a nonpolarized fashion. This result indicated that both tails contain a basolateral sorting signal. We also showed that the targeting signal of tail 1 is tyrosine-independent in contrast to most basolateral signals identified so far (23).

In this study, we characterized the sorting signal in the cytoplasmic tail of MCP-BC1 in more detail. By analysis of MCP-BC1 mutants with deletions in the cytoplasmic tail, an important part of the basolateral targeting signal was localized in the four carboxyl-terminal amino acids (FTSL). Studies with point mutants revealed that in addition to the phenylalanine, the leucine residue at the very COOH-terminal end is also important. A mutant with the phenylalanine replaced by tyrosine was also transported to the basolateral surface of polarized epithelial cells; but in contrast to the wild type protein, it was efficiently endocytosed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**MDCK cells (strain I) were grown in Dulbecco's modified essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg of streptomycin. Tissue culture-treated 0.4-μm pore size Transwell polycarbonate filters (Costar Corp., Cambridge, MA) were used for all experiments. Cells were seeded 5 days before experiments (2 × 10^5 cells/7.5-mm unit and 2 × 10^5 cells/24-mm unit). The polarity was determined by measurement of the transepithelial resistance using a Millipore ERS apparatus (Bedford, MA). MDCK cells formed a tight monolayer with an electrical resistance of 1,000–2,500 MΩ cm^2. The different patterns of surface proteins on the apical and basolateral membrane of the polarized cell lines were controlled by surface biotinylation.

**Construction of MCP Mutants and Expression in MDCK Cells—**The construction of the two chimeric MCP proteins used in this study was described by Lublin and Coyne (24). The DAF-TM consists of amino acids 1–304 of decay-accelerating factor (DAF, CD55) and amino acids 270–350 of MCP-BC2, resulting in a DAF molecule anchored by the MCP transmembrane and cytoplasmic domain. The CD48-GPI (MCP-PI) consists of amino acids 1–269 of MCP and 307–347 of DAF (numbering starts from the first amino acid of the mature protein without the signal peptide), representing a GPI-anchored version of MCP (Fig. 1 upper panel). For our studies both chimeras were subcloned into the EcoRI site of the stable expression vector pH3Apr-neo (25). The sequences of the four cDNAs with larger deletions in the cytoplasmic tail of the isoform BC1 of MCP (tail-minus, d1–6, d7–12, and d13–16) was described by Liszewski et al. (26). For the generation of the cDNA mutants 13F/Y, 13F/A, d14–16, d16, and 16L/A, MCP isoform BC1 was subcloned into the replicative form DNA of M13mp18. Oligonucleotide-directed mutagenesis based on the phosphorothioate method using single-stranded DNA of M13mp18. Oligonucleotide-directed mutagenesis based on the phosphorothioate method using single-stranded DNA of M13mp18. Oligonucleotide-directed mutagenesis based on the phosphorothioate method using single-stranded DNA of M13mp18. Oligonucleotide-directed mutagenesis based on the phosphorothioate method using single-stranded DNA of M13mp18.

**Antibody Uptake Assay—**MDCK cells stably expressing MCP were clones grown on 7.5-mm Transwell units were fixed on ice with 2% paraformaldehyde (15 min). The apical and basolateral surfaces were incubated with mAb J4/48 and a fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (DAKO, Denmark) as described earlier (27). The fluorescence label was visualized with a confocal laser scanning microscope (LSM Carl Zeiss, Oberkochem, Germany) working with the blue line of an argon laser.

**Confocal Microscopy—**For surface immunofluorescence, MDCK cell clones grown on 7.5-mm Transwell units were fixed on ice with 2% paraformaldehyde (15 min). The apical and basolateral surfaces were incubated with mAb J4/48 and a fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (DAKO, Denmark) as described earlier (27). The fluorescence label was visualized with a confocal laser scanning microscope (LSM Carl Zeiss, Oberkochem, Germany). The blue line of an argon laser was used. Antibody uptake assay—MDCK cells stably expressing MCP were grown on coverslips. At 10–50% confluence, surface-expressed MCP was labeled with a mAb directed against MCP J4/48 (Dianova) diluted 1:20 in PBS-bovine serum albumin. After an incubation for 60 min on ice, the antibody was removed by washing with PBS, and the cells were incubated with cell culture medium for 60 min either at 4 °C or 37 °C to allow endocytosis of the MCP-antibody complex. Internalization was stopped by rapid cooling on ice. Surface-bound antibody was detected by an incubation for 60 min on ice with a rhodamine-conjugated goat anti-mouse Fab fragments (Sigma) at a dilution of 1:100 in PBS-bovine serum albumin. After washing with PBS, the cells were fixed and permeabilized for 5 min at −20 °C with methanol/acetone.

**FIG. 1.** Diagram of MCP-DAF chimeras and amino acid sequences of the cytoplasmic domains of MCP isoform BC1 mutants. In the upper panel a diagram of the MCP, DAF-TM, and the MCP-PI chimeras is shown. The ectodomain of MCP consists of the four SCRs, the STP B and C, and a short region of unknown function (U). The TM domain is followed by an intracytoplasmic anchor (IA) and the cytoplasmic domain (CT). The DAF-TM comprises the four SCRs (SCR) and the O-linked carbohydrate domain (OD) of DAF (AS 1–304) followed by the TM domain, the intracytoplasmic anchor, and the cytoplasmic domain of MCP-BC2 (AS 270–350). The MCP-PI comprises the four SCRs and the STP B of MCP (AS 1–269) followed by a GPI that results from the cleavage of the DAF-GPI anchoring hydrophobic signal encoded in amino acids 307–347 of DAF. In the lower panel the end of the TM domain, the intracytoplasmic anchor, and the cytoplasmic domains of MCP isoform BC1 wild type (BC1-wt), and mutants are shown in a single letter amino acid code.

**Domain-specific Biotinylation of Cells Grown on Filters and Isolation of MCP—**Surfaces of filter-grown MDCK cells were labeled with the non-membrane-permeating reagent sulfo-N-hydroxysuccinimidylbietin (Pierce) as described recently (23).
(11). Internalized antibodies were detected with FITC-labeled rabbit anti-mouse IgG (DAKO) at a dilution of 1:500 in PBS-bovine serum albumin. To avoid nonspecific binding to the rhodamine-labeled goat Fab fragments bound to the cell surface, the FITC-labeled antiseraum was preabsorbed with goat-IgG agarose (Sigma). After the double immunofluorescence staining, the samples were mounted in Mowiol and 10% triethylenediamine. Conventional epifluorescence was performed with an Axiohot microscope (Zeiss). Pictures were taken with Kodak Tmax film (3200 ASA) exposed for identical times for both fluorochromes.

**Sialidase Protection Assay—**MDCK cells expressing MCP were grown on six-well plastic dishes and surface labeled at 4 °C with sialic acid as described earlier (27). After stopping the sialylation reaction by adding PBS− containing 0.1 m glycine and washing with cold PBS−, prewarmed serum-free cell culture medium was added. The cells were transferred to a 37 °C incubator for various times. To stop internalization, the cells were cooled on ice and washed with cold PBS+. Subsequently, the cells were incubated with 500 milliunits Vibrio cholerae sialidase (VCNA; Behring, Marburg, Germany) for 60 min at 4 °C. This treatment removed all sialic acid residues on surface MCP, whereas internalized MCP was protected from the sialidase. After extensive washing with cold PBS+, the cells were lysed, and MCP was immunoprecipitated as described earlier (27). Biotinylated MCP was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose, and detected as described above. The internalization rate of MCP was determined by densitometric quantification of the sialylated and desialylated MCP bands.

**RESULTS**

**The Cytoplasmic Domain of MCP Contains a Basolateral Targeting Signal—**We have reported recently that the cytoplasmic tail of MCP is responsible for the basolateral transport of the isoforms BC1 and BC2 in polarized epithelial cells (23). To analyze whether the basolateral targeting signal of MCP is able to redirect an apical protein to the basolateral domain, two chimeric molecules (Fig. 1, upper panel) that have been constructed from MCP-BC2 and DAF (24) were stably expressed in MDCK cells. DAF is known to be localized on the apical surface of polarized cells (28). The first chimera was a form of DAF in which the GPI anchor was replaced by the MCP transmembrane and cytoplasmic domain (DAF-TM). The second chimera represents a form of MCP which lacked both the transmembrane and the cytoplasmic domain and was anchored by GPI (MCP-PI).

To analyze the transport of these proteins, MDCK cells stably expressing either of the chimeras were grown on permeable filter supports. Either the apical or the basolateral surface proteins were labeled by adding the non-membrane-permeating reagent sulfo-N-hydroxysuccinimidylbiotin to the respective chamber. The cells were lysed, and MCP and DAF were isolated by immunoprecipitation (mAb J4/48 for MCP, anti-mouse IgG for DAF). The samples were divided into two aliquots. One was used for a Western blot analysis to ensure that there was no difference in the total amount of protein in the cells labeled from two different sides (not shown). The second aliquot was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, blotted to nitrocellulose, and the biotin-labeled proteins were detected with streptavidin/peroxidase. As shown in Fig. 2, MCP and MCP chimeras were distributed in a different way on epithelial cells. MCP-BC1 representing a wild type isoform is almost exclusively expressed on the basolateral surface as described recently (23). DAF-TM was also found predominantly on the basolateral side indicating that the MCP COOH terminus comprising the transmembrane and the cytoplasmic domain is able to direct an apical protein to the basolateral membrane of polarized cells. In contrast to DAF-TM, an efficient biotinylation of the MCP-PI molecules was only obtained after labeling the cells from the apical side. Replacement of the COOH terminus of MCP by a GPI anchor resulted in the apical expression of the protein. This finding is in agreement with the apical localization of other GPI-anchored proteins.

**Deletion of the Four COOH-terminal Amino Acids FTSL**

**BC1-wt**

**DAF-TM**

**MCP-PI**

**tail-minus**

**BC1-d1-6**

**BC1-d7-12**

**BC1-d13-16**

**Abolished the Basolateral Transport of MCP-BC1—**Our previous work indicated that the basolateral sorting signal of the BC1 isoform of MCP is localized in the 16 amino acids of the cytoplasmic tail. Studies on a deletion mutant lacking the six membrane-proximal amino acids (TYLYLTD) indicated that the targeting signal is not dependent on the only tyrosine of the cytoplasmic tail (23). Here, we analyzed two additional deletion mutants lacking either six amino acids in the central portion (BC1-d7–12) or four amino acids at the very COOH-terminal end of the cytoplasmic tail (BC1-d13–16). The amino acid sequences of the cytoplasmic portion of these and all mutants described in this paper are shown in Fig. 1 (lower panel). The distribution of the BC1 deletion mutants was analyzed by domain-specific surface biotinylation of stably expressing MDCK cells grown on filters. The result is shown in Fig. 2. As demonstrated recently (23), the MCP form lacking the cytoplasmic tail (tail-minus) was almost equally distributed on the apical and the basolateral membranes. The deletion mutants lacking either the first six amino acids or the following six amino acids (BC1-d1–6 and BC1-d7–12) were transported like the wild type protein (MCP-BC1). Therefore, the first 12 amino acids of the cytoplasmic tail are dispensable for the correct basolateral sorting of MCP. In contrast, the mutant lacking the four COOH-terminal amino acids of the tail (BC1-d13–16) was transported like the tail-minus mutant. This finding indicates that the basolateral sorting of MCP is dependent on the amino acids FTSL at the carboxyl terminus of the protein.

**Importance of the Phenylalanine for the Basolateral Targeting Signal—**Aromatic amino acids, especially tyrosine, are involved in the targeting of several basolateral proteins. As phenylalanine is the only aromatic amino acid in the FTSL motif of MCP, we analyzed the importance of this residue. We established MDCK cells stably expressing MCP-BC1 with the phenylalanine at position 13 of the cytoplasmic tail replaced either by tyrosine (13F/Y) or by alanine (13F/A). Mutant MCP forms were analyzed by surface biotinylation of filter-grown cells. The result of domain-specific biotinylation is shown in Fig. 3. Like the wild type protein (Fig. 2, MCP-BC1), 13F/Y was found mainly on the basolateral membrane. This finding indicates that phenylalanine in the basolateral targeting signal of MCP can be replaced by tyrosine without changing the direction of the transport. In contrast, substitution of an alanine for the phenylalanine altered the polarized transport dramatically. 13F/A was found to be equally distributed on both sides of the cells, similar to the BC1-d13–16 mutant. To confirm the different distribution of the 13F/Y and 13F/A mutants, the filter-grown cells were also analyzed by indirect immunofluorescence.
described as an endocytosis signal for several proteins (29, 30),

...amount of protein was comparable to BC1-wt. Because a motif protein BC1–13F/Y was strongly reduced although the total other mutants we tested, the amount of surface-expressed pro-

...MCP with the phenylalanine replaced by... in the cytoplasmic tail... in position 16 of the cytoplasmic tail replaced by a tyrosine (13F/Y) or by an alanine (13F/A) was expressed in filter-grown MDCK cells. After domain-specific biotinylation from the apical (a) or the basolateral (b) side, respectively, the cells were lysed, and MCP was immunoprecipitated by mAb J4/48. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized after transfer to nitrocellulose by incubation with streptavidin/peroxidase.

FIG. 3. Surface expression of MCP-BC1 with an exchange of the phenylalanine in the cytoplasmic tail. MCP-BC1 cDNA with the phenylalanine in position 13 of the cytoplasmic tail replaced by a tyrosine (13F/Y) or by an alanine (13F/A) was expressed in filter-grown MDCK cells. After domain-specific biotinylation from the apical (a) or the basolateral (b) side, respectively, the cells were lysed, and MCP was immunoprecipitated by mAb J4/48. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized after transfer to nitrocellulose by incubation with streptavidin/peroxidase.

FIG. 3. Surface expression of MCP-BC1 with an exchange of the phenylalanine in the cytoplasmic tail. MCP-BC1 cDNA with the phenylalanine in position 13 of the cytoplasmic tail replaced by a tyrosine (13F/Y) or by an alanine (13F/A) was expressed in filter-grown MDCK cells. After domain-specific biotinylation from the apical (a) or the basolateral (b) side, respectively, the cells were lysed, and MCP was immunoprecipitated by mAb J4/48. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized after transfer to nitrocellulose by incubation with streptavidin/peroxidase.

The COOH-terminal Leucine Is Important for the Basolateral Targeting of MCP—To analyze further the basolateral targeting of MCP, three mutants with changes in the three COOH-terminal amino acids (TSL) were generated. MCP lacking all three amino acids (d14–16) or only lacking the leucine (d16) and a MCP form with a substitution of an alanine for the leucine (16L/A) were stably expressed in MDCK cells and analyzed by surface biotinylation and confocal microscopy of filter-grown cells. As shown in Fig. 5, the three mutants were almost equally distributed on the surface of MDCK cells, indicating that removal or substitution of the leucine in position 16 of the cytoplasmic tail results in the loss of the polarized transport. The confocal immunofluorescence micrographs of BC1-d14–16, BC1-d16, and BC1-16L/A are shown in Fig. 6. Fluorescence signals were detected in all sections (apical, center, and basal) of MDCK cells expressing any of the three mutants. This result confirms the result of the domain-specific biotinylation and indicates that in addition to the phenylalanine at position 13 the leucine at position 16 is also essential for the correct sorting of MCP.

Replacement of Phenylalanine by Tyrosine Generates an Endocytosis Signal—MCP with the phenylalanine replaced by tyrosine (BC1–13F/Y) was transported to the basolateral membrane like the wild type protein (BC1-wt). In contrast to all other mutants we tested, the amount of surface-expressed protein BC1–13F/Y was strongly reduced although the total amount of protein was comparable to BC1-wt. Because a motif (YXXL) similar to that one generated in this mutant (YTSL) is described as an endocytosis signal for several proteins (29, 30),... using a confocal laser scanning microscope. After cell fixation with paraformaldehyde, the nonpermeabilized cells were incubated from both the apical and the basolateral side with mAb J4/48 and a FITC-conjugated anti-mouse immunoglobulin. Horizontal sections of the apical, the center, and the basal portions of the cells are shown in Fig. 4 (upper panel). In addition, a vertical section (side view) of the cells is shown in the bottom panel of Fig. 4. Almost no fluorescence signals were detected in the apical section of MDCK cells expressing BC1–13F/Y. The section through the center of the cells showed a honeycomb pattern that in a more diffuse manner was also seen in the basal section. In the side view, a cup-like pattern typical for basolateral proteins was observed. In contrast, strong fluorescence signals were found in all sections of MDCK cells expressing BC1–13F/A. In the vertical profile, BC1–13F/A was found to encircle the cells completely. The confocal immunofluorescence analysis confirmed the result that replacement of the phenylalanine by a tyrosine has no effect on the targeting of MCP to the basolateral surface of polarized cells, whereas the replacement by an alanine resulted in the loss of polarized surface expression.

FIG. 4. Confocal immunofluorescence microscopy of MCP-BC1 with an exchange of the phenylalanine in the cytoplasmic tail. MDCK cells expressing MCP-BC1 with a tyrosine (13F/Y) or an alanine (13F/A) instead of a phenylalanine at position 13 of the cytoplasmic tail were grown on filters. After fixation with 2% paraformaldehyde, the apical and basolateral surfaces were incubated with mAb J4/48 and a FITC-conjugated second antibody. Analysis was performed with a laser scanning microscope. Confocal immunofluorescence micrographs of horizontal focal planes through the apical surface, the center, the basal surface, and a vertical profile (side view) through the monolayers are shown.

FIG. 5. Cell surface distribution of MCP-BC1 with mutations in the last three amino acids of the cytoplasmic tail. MDCK cells expressing MCP isoform BC1 lacking the last three amino acids of the cytoplasmic tail (d14–16), lacking the last amino acid (d16), or having the leucine at position 16 replaced by an alanine (16L/A), were analyzed for polarized surface distribution by domain-specific biotinylation: apical (a) or basolateral (b). the weak surface expression may have resulted from internalization of BC1–13F/Y. To examine this possibility, we analyzed BC1–13F/Y for endocytosis by an antibody uptake experiment. MCP at the surface of living cells was incubated with mAb J4/48 at 4 °C, and the cells were either kept on ice for 60 min or warmed to 37 °C to allow endocytosis to occur. Surface-bound antibodies were detected by incubation of the living cells with a rhodamine-conjugated second antibody at 4 °C. After permeabilization of the cells, internalized MCP-J4/48 complexes were detected with a FITC-conjugated second antibody. In Fig. 7, the result of the double immunofluorescence staining (surface, intracellular) is shown for wild type MCP (BC1-wt) and the tyrosine mutant (BC1–13F/Y). With cells maintained at 4 °C neither wild type nor mutant MCP was detected intracellularly. After incubation at 37 °C, most of the surface-bound J4/48 was still detectable on the surface of MDCK cells express-
BC1-13F/Y mutant analyzed by a sialidase protection assay. MDCK cells expressing MCP-BC1 wild type (BC1-wt) or MCP-BC1 with the phenylalanine at position 13 in the cytoplasmic tail replaced by a tyrosine (BC1–13F/Y) were grown to confluence. After surface biotinylation, the cells were incubated at 37 °C for 0, 5, 15, 30, or 50 min. Cells were treated at 4 °C without (−) or with (+) VCNA for 60 min. After lysis of the cells, MCP was immunoprecipitated. The samples were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. Biotinylated sialylated (+SA) and desialylated (−SA) MCP were detected by streptavidin/peroxidase.

BC1–13F/Y no fluorescence signals were seen after incubation at 37 °C. Almost all MCP was endocytosed and could be detected by intracellular staining. To confirm the internalization of BC1–13F/Y, we performed a sialidase protection assay. MCP-expressing cells were surface labeled with biotin and chased for various periods at 37 °C to allow internalization of proteins. The extent of endocytosis was measured by the proportion of biotinylated protein that became inaccessible to extracellular VCNA added at 4 °C at the end of the chase period. After digestion with VCNA, cells were lysed, and MCP was immunoprecipitated and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Biotinylated proteins were detected after transfer to nitrocellulose by streptavidin/peroxidase.

MCP on the cell surface was sensitive to VCNA treatment. The release of sialic acids resulted in an increased electrophoretic mobility (−SA). Internalized protein was resistant to the enzyme treatment and retained its sialic acids (+SA). In Fig. 8, a clear difference between BC1-wt and BC1–13F/Y can be observed. The protein bands were quantified by densitometric scanning to determine the endocytosis rate. In the case of BC1-wt, even after 50 min at 37 °C only desialylated protein was detectable, indicating that no internalization had occurred, and all of the protein was sensitive to VCNA. In contrast, BC1–13F/Y was found to become VCNA-resistant with increasing incubation time at 37 °C. After 15 min 50% of MCP was still sialylated. The amount internalized increased to more than 90% after an incubation period of 50 min. With longer endocytosis times, the total amount of BC1–13F/Y decreased, probably because of degradation of the internalized protein.

**DISCUSSION**

Our results demonstrate that the cytoplasmic tail 1 of MCP contains a basolateral targeting signal. MCP constructs lacking the COOH terminus were affected in a different way. Substitution of a GPI anchor for the transmembrane domain (MCP-PI) resulted in the apical delivery of the chimeric protein. This finding was not unexpected because transport to the apical membrane is a general feature of GPI-anchored proteins (14, 15). In contrast to GPI-anchored MCP, a mutant MCP lacking the cytoplasmic tail but retaining the transmembrane domain as well as the intracytoplasmic anchor was not redirected to the apical membrane but was transported to the cell surface in a nonpolarized fashion. This may indicate the lack of any targeting signal. For several other basolateral glycoproteins, it has been reported that deletion of the cytoplasmic tail results in the transport to the apical plasma membrane (7). N-Glycans have been suggested to be involved in the apical transport of glyco-
proteins (13). If carbohydrates indeed serve as apical targeting signals, one would have to postulate that the tail-minus mutant of MCP has retained a weak basolateral sorting signal that counteracts this apical targeting signal resulting in nonpolarized transport. However, experimental evidence for a role of N-glycans as apical targeting signal is available so far only for secretory proteins (31), not for transmembrane proteins. In this context it should be noted that nonpolarized transport has been observed not only for the MCP mutant but also for other basolateral proteins after inactivation of the basolateral targeting signal or after deletion of the cytoplasmic tail, e.g. for the asialoglycoprotein receptor (32) and the G protein of vesicular stomatitis virus (30).

Using deletion analysis, we have shown that the main targeting information of MCP-BC1 is contained within the amino acids FTSL at the COOH terminus of the protein. This sorting signal has similarity to the targeting determinants of several other basolateral proteins that follow the general motif XXX-large hydrophobic, e.g. the vesicular stomatitis virus G protein (30), the human nerve growth factor receptor (9), and the asialoglycoprotein receptor (32). The basolateral proteins described so far contain a tyrosine as critical aromatic amino acid, and their sorting signals are located within the cytoplasmic tail. The targeting motif of MCP compared with the basolateral proteins has two unique features: (i) it contains a phenyalanine at position 1 of the tetrapeptide; (ii) it is exposed at the end of the cytosolic domain. In agreement with the general motif, we found that neither phenyalanine nor leucine can be replaced by alanine without affecting the polarized transport of MCP. On the other hand, substitution of a tyrosine for the phenyalanine did not abolish the transport to the basolateral surface, indicating that phenyalanine and tyrosine can have the same function in the sorting event from the trans-Golgi network to the basolateral plasma membrane. Furthermore, our results demonstrate that a basolateral sorting signal can be recognized also when it is located in a terminal position.

All basolateral receptor proteins studied so far are endocytosed. Some of them are transcytosed to the apical cell membrane (33). The majority of endocytotic receptors are recycled to the plasma membrane and finally degraded in lysosomes with half-lives of 6–60 h (34). Lysosomal and Golgi proteins that are found transiently on the basolateral membrane are rapidly endocytosed and delivered to lysosomes and the trans-Golgi network, respectively (29, 35). Three types of signals for receptor-mediated endocytosis are known. Among these are a dileucine motif (36) and a terminal KXXX motif (37). The majority of all known internalization signals contain the motif aromatic-XX-large hydrophobic. In most cases the aromatic residue is a tyrosine, and therefore the signal overlaps with the basolateral targeting signal mentioned above, e.g. in the lysosomal acid phosphatase (10) and in the asialoglycoprotein receptor (32). We found that MCP-BC1 is endocytosed only to a very low extent. In the antibody uptake experiment a very small amount of MCP was visible intracellularly, whereas no endocytosed protein was detectable in the sialidase protection assay. This may be caused by a lower sensitivity of the sialidase assay or clustering of MCP in coated pits after antibody binding, resulting in an enhanced internalization. Such a low internalization rate has not been described for any other well characterized basolateral protein. In this respect, MCP resembles the apically expressed influenza hemagglutinin, which has been shown to be excluded from coated pits and which is internalized 40 times more slowly than is the bulk of the plasma membrane (38). The structural basis for the inefficient endocytosis of HA is, in addition to the lack of a tyrosine-dependent signal in the cytoplasmic tail, a structural feature within the transmembrane domain (39). Whether the transmembrane domain of MCP contributes to the inefficient internalization of this protein remains to be shown. The cytoplasmatic tail of MCP-BC1 obviously does not contain an endocytosis signal, although a tyrosine is present at position 2 of the tail sequence. The FTSL sequence that is important for the basolateral targeting is not sufficient for endocytic uptake. The reason for this is not the terminal position of this tetrapeptide but the aromatic amino acid at position 1. By replacing the phenyalanine residue by a tyrosine, we created an efficient endocytosis signal. About 90% of BC1–13F/Y was endocytosed within 50 min. The internalization did not reach a plateau after 10–15 min, as is commonly observed with proteins that recycle to the plasma membrane, suggesting that the protein is delivered to lysosomes and degraded. This assumption is consistent with the observed decrease in the total amount of MCP during longer internalization times. In agreement with the rapid endocytosis, we detected only small amounts of BC1–13F/Y on the cell surface when we analyzed the steady-state distribution.

MCP provides an interesting example of how the targeting motif can be modulated to favor transport to the plasma membrane over endocytosis. The phenyalanine residue of the COOH-terminal FTSL sequence allows basolateral transport of MCP but avoids endocytosis. This is optimal for the physiological function of MCP, i.e. the binding of the complement factors C3b and C4b on the cell surface to prevent complement activation. As complement activation of the alternative pathway occurs continually, protection of the autologous cells from complement-mediated lysis requires the constitutive expression of protective proteins on the cell surface. Additionally, if MCP engages a ligand that is covalently bound to other membrane constituents, internalization would likely not be possible. Therefore, there need to be signals that take the protein to its proper location and prevent it from being internalized. The FTSL motif is an effective way to meet this requirement because it allows MCP to stay for extended periods of time on the basolateral surface, i.e. on the membrane domain that faces the serosal compartment. The correlation of MCP expression levels and cytoprotection was demonstrated by Oglesby et al. (41): mouse cells transfected with MCP were protected from complement in a dose-dependent manner. An example of the effect of low MCP surface expression is provided by measles virus-infected cells. Infection by certain strains of measles virus causes a rapid down-regulation of MCP, and this has been shown to result in an increased susceptibility of the cells for complement-mediated lysis (42).

Acknowledgment—We acknowledge gratefully the technical assistance of A. Heiner.

REFERENCES

1. Tucker, S. P., and Compans, R. W. (1993) Adv. Virus Res. 42, 187–247
2. Fuller, S. D., Bravo, R., and Simons, K. (1985) EMBO J. 4, 297–307
3. Griffiths, G., and Simons, K. (1986) Science 234, 438–443
4. Rindler, M. J., Ivanov, I. E., Plesken, H., Rodriguez-Boulan, E., and Sabatini, D. D. (1984) J. Cell Biol. 96, 1304–1319
5. Wandinger-Ness, A., Bennett, M. K., Antony, C., and Simons, K. (1990) J. Cell Biol. 111, 987–1000
6. Bartles, J. R., Feracci, H. M., Stieger, B., and Hubbard, A. L. (1987) J. Cell Biol. 105, 1241–1251
7. Mutter, K., Yamamoto, E. M., and Mellman, I. (1994) J. Cell Biol. 126, 991–1004
8. Fuhrer, C., Geffen, I., and Spiess, M. (1991) J. Cell Biol. 114, 423–431
9. Le Rivie, A., Samhuy, V., Patzak, A., Patil, N., Chao, M., and Rodriguez-Boulan, E. (1991) J. Cell Biol. 115, 607–618
10. Prill, V., Lehmann, L., von-Figura, K., and Peters, C. (1993) EMBO J. 12, 2181–2193
11. Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991) Cell 66, 65–75
12. Hunziker, W., Harter, C., Mutter, K., and Mellman, I. (1991) Cell 66, 907–920
13. Fiedler, K., and Simons, K. (1995) Cell 81, 309–312
14. Lisanti, M. P., Sargiacomo, M., Graeve, I., Salid, A. R., and Rodriguez-Boulan, E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9557–9561
15. Brown, D. A., Crise, I. W., and Rose, J. K. (1989) Science 245, 1499–1501
16. van’t Hof, W., Rodriguez-Boulan, E., and Memon, A. K. (1986) J. Biol. Chem. 261, 1241–1251
27. Liszewski, M. K., Tedja, I., Atkinson, J. P., and Herrler, G. (1994) J. Biol. Chem. 269, 10776–10779
28. Lisanti, M. P., Caras, I. W., Davitz, M. A., and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2145–2156
29. Humphrey, J. S., Peters, P. J., Yuan, L. C., and Bonifacino, J. S. (1993) J. Cell Biol. 120, 1123–1135
30. Thomas, D. C., and Roth, M. G. (1994) J. Biol. Chem. 269, 15732–15739
31. Scheiffele, P., Pera¨nen, J., and Simons, K. (1995) Nature 378, 96–98
32. Geffen, I., Fuhrer, C., Leitinger, B., Weiss, M., Huggel, K., Griffiths, G., and Spiess, M. (1993) J. Biol. Chem. 268, 20772–20777
33. Mostov, K. E., and Simister, N. E. (1985) Cell 43, 389–390
34. Braun, M., Waheed, A., and von Figura, K. (1989) EMBO J. 8, 3633–3640
35. Kernfeld, S., and Mellman, I. (1989) Annu. Rev. Cell Biol. 5, 483–525
36. Letourneur, F., and Klausner, R. D. (1992) Cell 69, 1143–1157
37. Itin, C., Kappeler, F., Linstedt, A. D., and Hauri, H. P. (1995) EMBO J. 14, 2250–2256
38. Roth, M. G., Doyle, C., Sambrook, J., and Gething, M.-J. (1986) J. Cell Biol. 103, 2607–2618
39. Lazarovits, J., Naim, H. Y., Rodriguez, A. C., Wang, R. H., Fire, E., Bird, C., Henis, Y. I., and Roth, M. G. (1996) J. Cell Biol. 134, 339–348
40. Deleted in proof
41. Oglesby, T. J., Allen, C. J., Liszewski, M. K., White, D. J. G., and Atkinson, J. P. (1992) J. Exp. Med. 175, 1547–1551
42. Schnorr, J.-J., Dunster, L. M., Nanan, R., Schneider-Schaules, J., Schneider-Schaules, S., and ter Meulen, V. (1995) Eur. J. Immunol. 25, 976–984
