Characterization of the Endosomal Sorting Signal of the Cation-dependent Mannose 6-Phosphate Receptor*

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Intracellular cycling of the cation-dependent mannose 6-phosphate receptor (CD-MPR) between different compartments is directed by signals localized in its cytoplasmic tail. A di-aromatic motif (Phe18-Trp19 with Trp19 as the key residue) in its cytoplasmic tail is required for the sorting of the receptor from late endosomes back to the Golgi apparatus. However, the cation-independent mannose 6-phosphate receptor (CI-MPR) lacks such a di-aromatic motif. Therefore the ability of amino acids other than aromatic residues to replace Trp19 in the CD-MPR cytoplasmic tail was tested. Mutant constructs with bulky hydrophobic residues (valine, isoleucine, or leucine) instead of Trp19 exhibited 30–60% decreases in binding to the tail interacting protein of 47 kDa (Tip47), a protein mediating this transport step, and partially prevented receptor delivery to lysosomes. Decreasing hydrophobicity of residues at position 19 resulted in further impairment of Tip47 binding and an increase of receptor accumulation in lysosomes. Intriguingly, mutants mislocalized to lysosomes did not completely co-localize with a lysosomal membrane protein, which might suggest the presence of subdomains within lysosomes. These data indicate that sorting of the CD-MPR in late endosomes requires a distinct di-aromatic motif with only limited possibilities for variations, in contrast to the CI-MPR, which seems to require a putative loop (Pro40-Pro-Ala-Pro-Arg-Pro-Gly45) along with additional hydrophobic residues in the cytoplasmic tail. This raises the possibility of two separate binding sites on Tip47 because both receptors require binding to Tip47 for endosomal sorting.

The biogenesis of lysosomes depends on the correct sorting of acid hydrolases from their place of synthesis in the endoplasmic reticulum to their final destination in lysosomes. Newly synthesized enzymes are tagged with a mannose 6-phosphate recognition marker by the concerted action of two enzymes: one which transfers a GlcNAc-1-phosphate to specific mannose residues on the acid hydrolases and the other which removes the covering GlcNAc to generate the mannose 6-phosphate tag (1, 2). This tag is required for the subsequent binding of the lysosomal hydrolases to one of two mannose 6-phosphate receptors (MPRs)1 in the trans-Golgi network (TGN). The receptor ligand complexes then exit the TGN in clathrin-coated vesicles and fuse with acidified endosomes. Following the pH-induced dissociation of the complexes, the lysosomal enzymes are further packaged into the lysosomes whereas the receptors are recycled back to the TGN to mediate another round of sorting (3, 4).

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a type I integral membrane protein with a C-terminal cytoplasmic tail of 67 residues. Within this cytoplasmic tail, several signals have been identified that direct the intracellular trafficking of the receptor. Internalization from the plasma membrane is mediated by 3 independent signals, a pair of phenylalanine residues (Phe13-X-X-X-X-Phe18), a classical tyrosine motif (Tyr45-X-X-Val48), and as indicated by in vitro data by a di-leucine based motif (Leu64-Leu65) (5, 6). The di-leucine motif has been shown in addition to mediate the transport of the CD-MPR from the TGN to the endosomes by interaction with Golgi-localized, γ-ear-containing, adenosine diphosphate ribosylation factor-binding protein 1 and adaptor protein complex 1 (AP1) and possibly also a sorting event within endosomes (7, 8). An acidic cluster as part of a casein kinase II site was implicated to be important for sorting in endosomes (9). Furthermore, overlapping with one of the internalization motifs (Phe13-X-X-X-X-Phe18) is a di-aromatic motif (Phe18-Trp19), which is required for the sorting of the receptor from late endosomes back to the TGN and therefore preventing degradation of the receptor in lysosomes (10). The sorting efficiency of the di-aromatic motif seems to be regulated by a post-translational modification, the reversible palmitoylation of Cys34 (10, 11).

Sorting of the receptor requires the interaction of the signals in the cytoplasmic tail with components of the cytoplasmic sorting machinery at sites of vesicle formation. Two such factors are the clathrin-associated adaptor complexes AP1 and adaptor protein complex 2 (AP2). AP1 mediates the sorting step from the TGN to endosomes and in addition recent data suggest that AP1 might also play a role in recycling MPRs from early endosomes to the TGN (12), whereas AP2 is required for rapid internalization of the receptor from the plasma membrane (13). Another protein, phosphofurin acidic cluster sorting protein 1 (PACS-1), was shown to interact with the receptor tail and AP1 in vitro suggesting that PACS-1 and AP1 may cooperate to sort MPRs (14). Most recently, the novel family of Golgi-localized, γ-ear-containing, adenosine diphosphate ribosylation factor-binding proteins (GGA) have been shown to mediate the sorting of MPRs from the TGN to endosomes (15, 16).

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1 The abbreviations used are: MPR, mannose 6-phosphate receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; TGN, trans-Golgi network; AP, adaptor protein complex; PNS, post-nuclear supernatant; Tip47, tail interacting protein of 47 kDa.
16). Yet another sorting event, from late endosomes back to the TGN, is mediated by tail interacting protein of 47 kDa (Tip47), which interacts with the di-aromatic motif (Phe19-Trp19) of the CD-MPR (17). Optimal Tip47 function depends on binding to Rab9, a late endosomal GTPase previously implicated in MPR retrieval, further confirming the role of Tip47 in this particular sorting event (18). Interestingly Tip47 binds to both MPRs, the CD-MPR and the cation-independent mannose 6-phosphate receptor (CI-MPR) but the CI-MPR does not contain a di-aromatic motif. A mutational analysis therefore showed that Tip47 seems to bind GagB (Galb, Switzerland). Goat antibodies were generously provided by Drs. D. Messner and M. Fukuda, respectively.

In this study we have analyzed the effect of mutations that change the key residue of the di-aromatic motif (Tip19) to bulky hydrophobic residues, non-polar residues, polar residues, or charged residues. Initial screening for the intracellular localization of CD-MPR constructs containing those mutations by immunofluorescence revealed some differences in the extent of co-localization with Lamp1, a lysosomal marker. To obtain a more quantitative measure for the effect of various mutations we tested their ability to interact with N-terminal His-tagged Tip47 in an in vitro pull-down assay. Constructs with Tip19 replaced by hydrophobic residues such as leucine and valine exhibited a modest impairment of binding to Tip47. However, correct endosomal sorting of the full-length CD-MPR in vivo was only achieved by wild-type receptors whereas the mutants showed an increased misorting to lysosomes by a factor of 5 for replacements of Tip19 by hydrophobic residues and up to a factor of 12 for replacements by charged residues.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes used in molecular cloning were obtained from Roche Diagnostics, New England Biolabs, or Promega; α-mercapto-ethanol, peroxidase inhibitors from Sigma; ECL Western blotting reagents from PerkinElmer Life Sciences; and cell culture dishes from Falcon. Oligonucleotides were synthesized either by the DNA synthesis facility of the Friedrich Miescher Institute or by Microchips GmbH (Switzerland). Goat antibodies and goat-anti mouse Alexa 488 antibodies were obtained from Molecular Probes (Eugene, OR). The mAb 22D4 specific for the bovine CD-MPR and 931A polyclonal antibodies specific for Lamp1 were generously provided by Drs. D. Messner and M. Fukuda, respectively.

**Recombinant DNA—**All basic DNA procedures were described as follows (20). The PCR procedure of Ho et al. (21) was used to generate the mutant constructs with pBSK-MPR TMD/tail (22) serving as a template together with bp 170–193 and 1260–1341 of pBSK—as the downstream and upstream primers, respectively. Appropriate partial complementary pairs of oligonucleotides in which the desired amino acid replacement had been incorporated were chosen as internal primers. The final PCR products were subcloned into pSFFV-neo as described (22). Human Tip47 was cloned by PCR from a Marathon Human Placental cDNA library (Clontech, Palo Alto, CA) using Tip47 down (5′-TTTGAATTCGCGCCGCTCTGTCGACC-3′) and Tip47 up (5′-GAGACCGCGCTCCTCTCTCC-3′) primers respectively. The PCR product was cut with BamHI and ligated into pSFFV-neo as described (20). The PCR product of human Tip47 was used as a template for a PCR with the oligos BlmHIpQE31-TIP47 down (5′-ACGGGATCCATGTTGCACGAC-3′) and SacI up (5′-TCCCTCTCCCTGCCCCCTGCCCCG-3′). The PCR product was cut with BamHI and SacI and ligated into pQE31 (Qiagen, AG, Basel). All coding sequences created by PCR were verified by sequencing. (Qiagen AG, Basel). All coding sequences created by PCR were verified together with bp 170–193 and 1260–1341 of pBSK as the downstream and upstream primers respectively. Appropriate partial complementary primers were generously provided by Drs. D. Messner and M. Fukuda, respectively.

**Cell Culture and Transfection—**HeLa cells were grown in α-minimal essential medium to 30% confluency before transfection with 1 μg of DNA using FuGENE according to the manufacturer's directions. The cells were incubated with 100 μl each of peptatin A and leupeptin 24 h before the experiment and analyzed 48 h post-transfection by confocal microscopy.

A mannose-6-phosphate-like growth factor II receptor-deficient mouse L cell line designated D9 (LRe−) was maintained in α-minimal essential medium. The cells were transfected with XbaI-linearized DNA with Lipofectin according to the manufacturer’s directions. Selection for resistance to neomycin (G418) was carried out using 500 μg/ml G418 as the final concentration. Resistant colonies were picked individually and screened for the expression of bovine CD-MPR by immunoblotting. Clones expressing similar amounts of receptor were expanded for further study and maintained in selective medium.

**Confocal Immunofluorescence Microscopy and Deconvolution—**Confocal immunofluorescence microscopy was carried out as described (23). Paraformaldehyde-fixed and saponin-permeabilized cells were incubated with 1:200 dilution of monoclonal antibody 22D4 and a 1:400 dilution of Ab 931A. The secondary antibodies were goat anti-rabbit Alexa 488 antibodies and goat-anti mouse Alexa 568 conjugates, diluted 1:1000. The coverslips were mounted on glass slides with ProLong Antifade (Molecular Probes) for viewing with an Olympus Fluoview FV500 confocal laser-scanning microscope. Serial sections in the z axis through the entire cells were taken, and the resulting stacks of images were processed on a multiprocessor SGI UNIX computer using the Huygens program (Scientific Volume Imaging, Hilversum, The Netherlands). A maximum likelihood estimation-based algorithm was used for image reconstruction. Z-stacks were exported as Tiff files and analyzed with the ImageJ program (R. W. H. Schindelin, Arizona, USA). In Vitro Pull-down Experiments—N-terminal His-tagged Tip47 was expressed in Escherichia coli strain M15. A saturated overnight culture was diluted 1:10 in 200 ml of growth medium and incubated for 1 h at 37 °C before induction with 1.0 mM isopropyl-thio-β-galactopyranoside for 2 h. The cells were then harvested by centrifugation, washed with ice-cold phosphate-buffered saline, and lysed by sonication in 20 ml of binding buffer (20 mM NaH2PO4, pH 7.8, 500 mM NaCl) containing a 1:500 dilution of a protease inhibitor mixture (5 mg/ml benzamidine, and 1 μg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 0.05% dimethyl sulfoxide, 0.50% ethanol) (PIC) and phenylmethylsulfonyl fluoride (40 μg/ml) (final concentration). Insoluble material was removed by centrifugation at 12,000 rpm for 1 h at 4 °C in a Sorvall GSA centrifuge. 4 ml of the supernatant was incubated for 30 min at room temperature on a rotating shaker with 400 μl of Ni-CAM HC resin (50% suspension) (Sigma) that was prewashed with binding buffer and blocked with binding buffer containing 0.1% bovine serum albumin. The beads were washed 2 times with wash buffer (binding buffer at pH 6.0) and once with assay buffer (50 mM HEPEs, 150 mM KCl, 5 mM imidazole, 1 mM MgCl2, pH 7.5) by spinning them at 1000 rpm for 1 min in a Heraeus centrifuge. Extracts from mouse D9 (LRe−) cells expressing wild-type or mutant CD-MPR constructs were prepared from cells grown on 15-cm Falcon tissue culture dishes in the following way. Confluent tissue culture dishes of cells were put on ice and rinsed with ice-cold phosphate-buffered saline, and lysed by sonication in 20 ml of binding buffer containing PIC and phenylmethylsulfonyl fluoride. The pellets were pelleted at 1000 rpm for 5 min at 4 °C in a Heraeus centrifuge and resuspended in 1 ml of assay buffer containing PIC and phenylmethylsulfonyl fluoride. The cells were homogenized in a ball bearing homogenizer of 51.2 μm clearance using 12 strokes and spun at 700 × g at 4 °C for 10 min. The supernatant was collected and centrifugation was repeated until no pellet appeared. The protein concentration of the post-nuclear supernatant (PNS) was measured using a BioRad protein assay. The appropriate amounts of PNS from each of the mutant cell lines and wild-type cell line expressing the same amount of CD-MPR were determined by Western blotting with anti-CD-MPR monoclonal antibody 931A followed by deconvolution using ImageQuant 5.0 software (Amersham Biosciences). These PNS amounts adjusted for expression levels were used for the assay.

Beads incubated with His6-Tip47 were resuspended in 200 μl of assay buffer containing PIC and phenylmethylsulfonyl fluoride, 50 μl of sample buffer added, and resuspended in the pCDA3.1(+ ) vector or an appropriate amount of PNS from mutant cell lines according to the expression level adjustment described above for 2 h at 4 °C on a rotating shaker. Beads were spun at 2500 rpm for 4 min at room temperature in an Eppendorf tabletop centrifuge. The supernatant was collected and stored. The beads were washed three times with sample buffer. Sample buffer was added to the beads at a final concentration of 40 μl, boiled, and analyzed by SDS-PAGE and Western blotting using anti-CD-MPR monoclonal antibody 22D4. An aliquot of each of the supernatants was also analyzed similarly to determine the amount of unbound receptor. The amount of CD-MPR bound to His6-Tip47 was...
determined by densitometric scanning using ImageQuant 5.0 software.

Percoll Gradient Fractionation—Confluent D9 (LRec−/H11002+) cells stably expressing wild-type or mutant forms of the CD-MPR were grown in a 100-mm Petri dish and incubated for 24 h in growth medium supplemented with 100 μM each of pepstatin A and leupeptin. The cells were then harvested, ruptured with a ball bearing homogenizer, and fractionated on 17.5% Percoll density gradients as described previously (22). The specificity of the lysosomal fractions was tested using a β-hexosaminidase assay as described previously (23) (data not shown).

SDS-PAGE and Immunoblotting—Proteins were separated on 10% SDS-polyacrylamide minigels (Bio-Rad) by using the Laemmli system (24). After electrophoresis gels were transferred onto nitrocellulose membranes according to the method of Towbin et al. (25). The immunoblotting was performed as previously described (22). The autoradiographs were quantitated by using a personal densitometer (Amersham Biosciences).

Assays and Miscellaneous Methods—β-Hexosaminidase activity was determined as described (22). Protein concentration was determined with the Bio-Rad protein assay kit by using protein standard I.

RESULTS

Intracellular Location of CD-MPR Wild-type Versus Mutant Forms—To determine whether the replacement of Trp19 in the cytoplasmic tail of the CD-MPR by various amino acids affects its ability to avoid delivery to lysosomes, wild-type and mutant constructs were prepared and expressed in HeLa cells. The cells were incubated for 24 h with pepstatin A and leupeptin to prevent degradation of receptor that was mislocalized to the lysosomes (23). After fixation with paraformaldehyde and permeabilization with saponin the cells were stained with antibodies against the CD-MPR and Lamp1, followed by the appropriate secondary antibodies. As expected, Lamp1 (Fig. 1, green) was localized in doughnut-shaped structures typical for the staining of a lysosomal membrane protein. In contrast, the CD-MPR wild-type (Fig. 1A, red) showed a predominant perinuclear staining representing localization in the TGN and late endosomes and, to a lesser extent, some peripheral staining that might represent early endosomes. There was very little colocalization of the two proteins indicated by the almost complete absence of yellow staining. The construct MPR-W19A was localized partially in lysosomes as indicated by an increased colabeling with Lamp1 compared with the CD-MPR wild-type (Fig. 1B). The amount of colabeling with Lamp1 was even further increased for the mutant MPR-W19D, demonstrating that a large percentage of this construct was delivered from endosomes to lysosomes instead of being recycled back to the TGN. Interestingly, although residing within the same structures the fluorescent signal for the CD-MPR mutant constructs was sometimes not filling the entire structure (Fig. 1, B and C, insets; yellow and green staining). Such a partial segregation would suggest distinct domains on continuous membranes.

These results demonstrate that the mutant receptors are not correctly sorted from the late endosomes back to the TGN and therefore accumulate in lysosomes. It also seems that the extent of missorting to lysosomes depends on the properties of the amino acid replacing Trp19 but this has to be characterized in more detail using a biochemical assay.

Hydrophobic Residues Poorly Replace Aromatic Residues at Position 19 in the CD-MPR Cytoplasmic Tail to Bind Tip47 in
Vitro—The immunofluorescence experiments suggested that the physical properties of the amino acid replacing Trp 19 determines the sorting efficiency of the CD-MPR mutants in late endosomes. Tip47 was shown to interact specifically with the di-aromatic motif of the MPRs and to mediate the transport from endosomes back to the TGN (17). Therefore we wanted to test the interaction of various CD-MPR mutants with Tip47 using an in vitro binding assay. Because of the potential effect of post-translational modifications we used the full-length receptor expressed in mammalian cells in contrast to previously published assays with truncated fusion proteins produced in E. coli. For this purpose we created a series of receptor constructs that consist of the full-length CD-MPR with various mutations of the residue Trp 19 (Fig. 2). Besides the replacements of Trp 19 with the bulky hydrophobic residues (Val, Ile, and Leu) we also used a less hydrophobic amino acid with a nonpolar side chain (Ala) as well as an amino acid with an uncharged polar side chain (Ser) or charged polar side chains (Arg and Asp). Post-nuclear supernatant from wild-type and mutant receptors expressed in mouse D9 cells (LRec/H11002) cells were incubated with His$_2$-Tip47 and incubated with His$_2$-Tip47 were incubated with either wild-type or mutant receptors in post-nuclear supernatant of mouse D9 cells for 2 h at 4 °C and washed three times to remove unbound proteins. Beads alone represents the negative control for the assay done by incubating wild-type receptor with beads without Tip47. Bound receptor was detected using SDS-PAGE and Western blotting with anti-CD-MPR antibody. Protein standard shows molecular mass assay done by incubating wild-type receptor with beads without Tip47. Unbound proteins. Beads alone represents the negative control for the efficiency of the assay was determined to be more than 20%. Quantitation was performed by densitometric scanning followed by ImageQuant 5.0.

Table I

<table>
<thead>
<tr>
<th>Construct</th>
<th>Percentage in lysosomes$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-MPR</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>MPR-FF</td>
<td>5 ± 3*</td>
</tr>
<tr>
<td>MPR-FY</td>
<td>4 ± 2*</td>
</tr>
<tr>
<td>MPR-YF</td>
<td>8 ± 5*</td>
</tr>
<tr>
<td>MPR-YW</td>
<td>3 ± 2*</td>
</tr>
<tr>
<td>MPR-W19V</td>
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<tr>
<td>MPR-W19I</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>MPR-W19L</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>MPR-W19C</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>MPR-W19A</td>
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</tr>
<tr>
<td>MPR-W19M</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>MPR-W19S</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>MPR-W19R</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>MPRW19D</td>
<td>59 ± 11</td>
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</tbody>
</table>

$^a$ Percentage of the various mutant receptors recovered in dense lysosomes on Percoll density gradients as shown in Fig. 4. The values are expressed as mean ± S.E.

Because the Tip47 binding assay was performed in vitro with Tip47 in the context of a His-tagged fusion protein isolated from E. coli, an additional assay was performed to analyze the efficiency of mutant constructs to avoid lysosomal delivery in vitro. Mouse L cells stably expressing the wild-type and mutant receptors were preincubated for 24 h in the presence of pepstatin A and leupeptin to inhibit degradation of receptors that had entered lysosomes (22). The cells were then harvested, homogenized, and the lysosomes were separated on 17.5% isosmotic Percoll density gradients (22, 26). Under these conditions, dense lysosomes are recovered at the bottom of the gradient (pool I), whereas low density membranes including endosomes, Golgi apparatus, plasma membrane, and endoplasmic reticulum are found near the top of the gradient (pool III). Intermediate density membranes are recovered in pool II. The distribution of the receptor in the three pools was determined by SDS-PAGE followed by Western blotting (Fig. 4 and Table I for quantitation of multiple experiments). As reported previously, the wild-type CD-MPR was almost completely excluded from dense lysosomes (5% recovered in pool I) and replacement of the di-aromatic motif ( Phe$_{18}$,Trp$_{19}$) with other aromatic residues did not affect the distribution significantly (MPR-FF, 5%; MPR-FY, 4%; MPR-YF, 8%; and MPR YW, 3%) (10). Substitution of Trp$_{19}$ with bulky hydrophobic residues led to an accumulation of the mutant receptors in dense lysosomes to about 30% (MPR-W19V, 27%; MPR-W19I, 27%; and MPR-W19L, 29%). A slightly larger accumulation of mutant receptors in lysosomes was found with the mutants that contained less hydrophobic, nonpolar side chains as replacements for Trp$_{19}$ (MPR-W19A, 38%; and MPR-W19M, 40%). To our surprise we found that the mutant MPR-W19C with the polar side chain of a cysteine residue accumulated only to about 34% in dense lysosomes. However, according to Kyte and Doolittle (27) the

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**TABLE I**

Summary of results of Percoll density gradients

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$^a$ Percentage of the various mutant receptors recovered in dense lysosomes on Percoll density gradients as shown in Fig. 4. The values are expressed as mean ± S.E.
A cysteine residue with its polar side chain is more hydrophobic than an alanine or a methionine residue. We next examined the effect of mutating Trp19 to a hydrophilic residue (MPR-W19S) or even charged residues (MPR-W19R and MPR-W19D). All three mutant receptors accumulated more than 50% in dense lysosomes (MPR-W19S, 52%; W19R, 56%; and MPR-W19D, 59%) compatible with the hypothesis that only hydrophobic residues can replace to a small extent an aromatic residue of the di-aromatic motif.

**DISCUSSION**

The results presented in this study demonstrate that replacing the key residue of the di-aromatic motif within the cytoplasmic tail of the CD-MPR, Trp19, by hydrophobic residues maintains endosomal sorting only to a minor extent. Mutant constructs with hydrophilic or even charged residues instead of Trp19 were highly missorted to lysosomes, indicating that these replacements were not functional at all.

To accomplish their biological function, sorting of lysosomal enzymes from the TGN to endosomes, it is essential that the MPRs avoid delivery to lysosomes where they would be degraded. Therefore the sorting step in late endosomes, which is mediated by the di-aromatic motif (10), is crucial and needs to be well characterized. Diaz and Pfeffer (17) first demonstrated that Tip47 interacts with the CD-MPR through the di-aromatic motif and with the CI-MPR that is lacking a di-aromatic motif and subsequently identified the sequence PPAPRP within the cytoplasmic tail of the CI-MPR as part of a complex three-dimensional binding domain for Tip47 (19). This raised the question if the interaction between Tip47 and the CD-MPR is strictly limited to aromatic residues or if other amino acids can replace Trp19, the key residue of the di-aromatic motif.

Using immunofluorescence we found that there is an increasing colocalization of mutant receptors with the lysosomal membrane protein Lamp1 upon decreasing hydrophobicity of the amino acid replacing Trp19. Interestingly, there was even less overlap than expected between the wild-type CD-MPR and Lamp1 as both proteins are present to some extent in late endosomes (28). This might indicate that Lamp1 and the CD-MPR are in different subdomains within late endosomes. It would make perfect sense that proteins destined for the lysosomes would be separated from proteins that recycle back to the Golgi thus increasing the sorting efficiency of such a complex structure as the multivesicular late endosomes. In fact, it has been observed that even the CD-MPR and the CI-MPR are localized in separate subdomains within late endosomes (29). Mutant receptors that were delivered partially to lysosomes like MPR-W19A (38% in lysosomes) and MPR-W19D (59% in lysosomes) did show an increasingly overlapping staining with Lamp1, validating this method as a screening procedure to test for the sorting efficiency of the mutant receptors. Intriguingly, there seemed to be subdomains of lysosomes that were labeled with either one or both proteins. Actually the majority of the structures that were colabeled for CD-MPR mutants and Lamp1 did not show a uniform yellow labeling that would indicate an equal distribution of the proteins but rather an overlap with separate labeling. Such a pattern of segregation of proteins indicates the existence of subdomains on the lysosomal membrane. These subdomains may be specialized for degradation as in the case of CD-MPR mutants or for export as in the case of molecules such as Lamp1 (30–33). It has been shown by Traub et al. (36) that AP-2 and clathrin could be recruited to lysosomes in permeabilized cells in vitro indicating that clathrin-coated vesicles mediate this transport step. Contrary to the existing notion of lysosomes as terminal organelles...
in the secretory pathway our observation suggests in addition that a functional specialization of its subdomains might exist. To follow up on this would require a method with a higher resolution such as immunoelectron microscopy.

To determine the sorting efficiency in a more quantitative way we first analyzed the ability of CD-MPR to bind His-tagged Tip47 in an in vitro assay. As with the immunofluorescence experiments the binding efficiency decreased if Trp19 was replaced by decreasing hydrophobic residues. However, even the mutants with Trp19 substituted by bulky hydrophobic residues such as leucine, valine, and isoleucine were moderately reduced in their capacity to bind Tip47. Mutants with a less hydrophobic residue at position 19, like MPR-W19A, displayed only marginal Tip47 binding whereas charged residues prevented binding almost completely. Binding was almost not affected when the di-aromatic mutants MPR-FW-YF and MPR-W19Y were tested in the assay. This clearly demonstrates that optimal interaction of the CD-MPR cytoplasmic tail with Tip47 depends on the di-aromatic motif with only limited possibilities for variations. The strict requirement for individual amino acids is further emphasized by the fact that the cytosolic tail of CD-MPR is 100% identical in all mammalian species cloned so far with only two homologous substitutions (Val1-Ile6 and Asp36-Glu56) in lower vertebrates such as chicken (4, 34).

When we analyzed the mutants in vivo in the context of the full-length receptor for their efficiency to avoid delivery to lysosomes we found that there were roughly three categories. Substitution of Trp19 with either one of the bulky hydrophobic residues resulted in mislocalization of the mutant construct to the mutants accumulating in dense lysosomes (Fig. 4, Table I) that were sorted like wild-type receptor.

It is interesting that the CD-MPR has a clearly defined signal, the di-aromatic motif that is required for the interaction with Tip47 and the sorting in late endosomes, which seems to be in contrast to the CI-MPR that relies on a domain comprising residues 48–75 in its cytoplasmic tail, with additional residues required for the proper presentation (19). Nevertheless, proper presentation of the di-aromatic signal is also required as mutations that prevent the reversible palmitoylation of the CD-MPR lead to a missorting to lysosomes (11). Clearly, further studies are required to determine whether both receptors have the same binding site on Tip47 or if there are two different binding sites. However, these experiments will be technically demanding because the receptors are membrane proteins that undergo important post-translational modifications such as palmitoylation.

Acknowledgments—We thank Drs. D. Messner and M. Fukuda for kindly providing antibodies against the CD-MPR and Lamp1, respectively. Special thanks to Dean Flanders from the IT group of the Friedrich Miescher Institute who provided us with enough computer power to run the deconvolution software for the immunofluorescence pictures. We acknowledge Prof. Eric G. Berger for continuous support and critical reading of the manuscript.

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