A Di-aromatic Motif in the Cytosolic Tail of the Mannose Receptor Mediates Endosomal Sorting*

Received for publication, January 27, 2000, and in revised form, June 22, 2000
Published, JBC Papers in Press, July 14, 2000, DOI 10.1074/jbc.M000571200

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The mannose receptor (MR), the prototype of a new family of multilectin receptor proteins important in innate immunity, undergoes rapid internalization and recycling from the endosomal system back to the cell surface. Sorting of the MR in endosomes prevents the receptor from entering lysosomes where it would be degraded. Here, we focused on a diaromatic sequence (Tyr18-Phe19) in the MR cytoplasmic tail as an endosomal sorting signal. The subcellular distribution of chimeric constructs between the MR and the cation-dependent mannose 6-phosphate receptor was assessed by Percoll density gradients and cell surface assays. Unlike the wild type constructs, receptor with alanine substitutions of Tyr18-Phe19 were highly missorted to lysosomes, indicating that the di-aromatic motif of the MR cytoplasmic tail mediates sorting in endosomes. Within this sequence Tyr18 is the key residue with Phe19 contributing to this function. Moreover, Tyr18 was also found to be essential for internalization, consistent with the presence of overlapping signals for internalization and endosomal sorting in the cytosolic tail of the MR.

A di-aromatic amino acid sequence in the cytosolic tail has now shown to function in two receptors known to be internalized from the plasma membrane, the MR and the cation-dependent mannose 6-phosphate receptor. This feature therefore appears to be a general determinant for endosomal sorting.

The immune system consists of two functional subunits, the innate or nonadaptive immune response, which represents a first-line host defense, and the so-called adaptive or clonal immune response. Macrophages are key components in both innate and adaptive immunity. They are widely distributed throughout many organs where they form a lattice beneath epithelial surfaces to protect the ports of entry of infectious agents. This goal is achieved by a variety of cell surface receptors, which are able to recognize antigenic arrays or patterns that are generic to microorganisms (1). The best characterized pattern recognition receptor is the mannose receptor (MR), which recognizes patterns of carbohydrates that decorate the surfaces and cell walls of many pathogens. Preferred ligands are glycoproteins with terminal mannose and fucose, whereas N-acetylglucosamine and glucose bind with lower affinity (2, 3). In addition, ligand binding requires Ca²⁺ (4). The mature MR is composed of a single subunit, which has an apparent molecular mass of 180 kDa and bears N- and O-linked sugars (5–7). It is synthesized as an inactive precursor unable to bind ligands in the endoplasmic reticulum and becomes fully active only during transit through the Golgi apparatus (8). Cloning of the MR further revealed that it is a type I transmembrane protein, which consists of five different domains: an N-terminal cysteine-rich region, a fibronectin type II domain, a series of eight C-type lectin-like carbohydrate recognition domains, a single transmembrane domain, and a 45-amino acid cytoplasmic tail (5, 6). This overall topology is shared by three additional proteins, the phospholipase A2 receptor (9), DEC205, a receptor without known ligand found in dendritic cells (10), and a novel C-type lectin (11) that was recently shown to be a protein-associating protein in human U937 cells (12). The MR thus represents the prototype of a new family of multilectin receptor proteins (13) that are widely distributed in virtually all organs in various cell types.

Recent work has pointed to the presence of a signal transduction pathway associated with ligand binding to the MR. Especially studies with pathogens bearing surface-bound mannos (as well as mannosylated particles demonstrate that secretion of mediators and cytokines is activated via the MR (14–16). These new data raise fundamental questions about the biological role of the MR. In particular, the intracellular trafficking of the MR becomes an important issue for the regulation of its biological activity. Previous studies of the trafficking of the MR have demonstrated that the receptor mediates both rapid endocytosis and phagocytosis at the cell surface. MR-mediated phagocytosis has been found in macrophages (17–19) and in transfected COS cells (18) but the exact trafficking of the MR during phagocytosis is not well understood. In contrast, more is known about the endocytic route of the MR. Initially, ligands bind to the MRs at neutral pH at the plasma membrane. The receptor-ligand complexes subsequently enter acidified endosomal compartments where the lower pH causes dissociation of the ligands. To mediate repeated rounds of internalization, the MRs recycle constitutively from the endosomal system back to the plasma membrane (4, 17). Importantly they avoid being transported to lysosomes where they would be degraded (7, 20, 21). Both the cytosolic tail and the transmembrane domain are required for the endocytic function of the MR as demonstrated by analyzing chimeric constructs between the Fc receptor and the MR (18). In contrast, MRs lacking the cysteine-rich domain and the fibronectin type II factor II receptor; NHS-SS-biotin, sulfo-N-succinimidyl-2(biotinamido)ethyl-1,3-dithiopropionate.

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† Supported by National Institutes of Health Grant 2R01AI20015-16.
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§ The abbreviations used are: MR, mannose receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; TGN, trans-Golgi network; PCR, polymerase chain reaction; wt, wild type; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CI-MPR/IGFII receptor, cation-independent mannose 6-phosphate/insulin-like growth factor II receptor; NHS-SS-biotin, sulfo-N-succinimidyl-2(biotinamido)ethyl-1,3-dithiopropionate.
domain showed no loss of endocytic capacity when expressed in fibroblasts (22).

In the present paper we have elucidated the intracellular trafficking of the MR in more detail. To fulfill its biological role, it is essential for the MR to be correctly sorted in endosomes to remain in the endosome/plasma membrane recycling pathway and avoid transport to lysosomes with subsequent degradation. The trafficking of other recycling receptors is known to be regulated by sorting signals that are recognized at different stages of the pathway. In particular, the endosomal sorting step has recently been shown for the cation-dependent mannose 6-phosphate receptor (CD-MPR) to be directed by a di- aromatic amino acid motif (Phe18-Trp19) in its cytoplasmic tail (23). The CD-MPR is a type I integral membrane protein that functions to transport newly synthesized acid hydrolases from the trans-Golgi network (TGN) to endosomes thereby recycling between the TGN, endosomes, and the plasma membrane (24–26). By analyzing the intracellular distribution of a variety of chimeric constructs between the MR and the CD-MPR we demonstrated that, analogous to the CD-MPR, a pair of aromatic residues (Tyr18-Phe19) in the cytosolic tail of the MR mediates sorting in endosomes. This finding is consistent with the di- aromatic motif being a general determinant for this sorting step.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes used in molecular cloning were obtained from Roche Molecular Biochemicals, Bioinex (Praroman, Switzerland), or Life Technologies, Inc.; fetal calf serum and Lipofectin were from Life Technologies, Inc.; Percol was from Amersham Pharmacia Biotech; nitrocellulose from Schleicher & Schuell; protease inhibitors were from Sigma; ECL Western blotting reagents were from Amersham Pharmacia Biotech or NEN Life Science Products. Oligonucleotides were synthesized by the DNA synthesis facility of the Friedrich Miescher Institut.

**Recombinant DNA**—All basic DNA procedures were as described (27).

To create the MPR-MR chimeric constructs, BgII and MluI sites were engineered to flank the transmembrane domain and cytosolic tail of the MR by PCR. A human placenta QUICK-Clene cDNA (CLONTECH Laboratories Inc.) was used as a template together with the oligonucleotides AAAAGAGATCTTTCCCCAGGCCGAGCTAG (Bgl-MR.d) and GAAACCGTTGAGGTACTAGATGCCG (Mlu-MR.up) as downstream and upstream primers. In addition, the endogenous BgII site in the cytosolic tail was destroyed by a silent point mutation in and the purified fragment was assembled with the Eco (30). The cells were transfected with 4 100 µg/ml streptomycin at 37 °C in a 5% humidified CO2 atmosphere.

**Cell Culture and Transfection**—A cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor-deficient mouse L cell line designated D9 (LRec) was maintained in α-modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% humidified CO2 atmosphere (30). The cells were transfected with 4 µg of XbaI-linearized DNA using Lipofectin according to the manufacturer’s directions. Selection was performed with neomycin (G418), and resistant colonies were screened for expression by immunoblotting. Selected clones were expanded for further study and maintained in selective medium.

**Antibodies**—Monoclonal antibody 22D4 was generously provided by Dr. D. Messner (31). This monoclonal antibody is specific for the bovine CD-MPR and does not cross-react with the human CD-MPR.

**Percoll Gradient Fractionation**—Confluent cells grown in a 100-mm Petri dish were incubated for 24 h in growth medium supplemented with 100 µg 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 (28). In brief, an equal volume of the cell lysate was mixed with Percoll and loaded onto the top of the tube and then combined as follows: fractions 1–3 (pool I, containing 70–80% of lysosomal enzyme activity), fractions 4–6 (pool II, containing intermediate density membranes), and fractions 7–9 (pool III, containing low density membranes including endosomes, the Golgi complex, plasma membrane, and the endoplasmic reticulum). The Percoll was removed by three centrifugation steps and the pelleted membranes were solubilized in 0.5% Triton X-100. An aliquot corresponding to 1:10 of the total volume was removed for the β-hexosaminidase assay, and the remaining sample was boiled in nonreducing SDS sample buffer. Aliquots corresponding to 1:15 of the final sample volume were analyzed by SDS-PAGE and immunoblotting.

**SDS-PAGE and Immunoblotting**—Proteins were separated on 10% SDS-polyacrylamide minigels (Bio-Rad) by using the Laemmli system (32). After electrophoresis, gels were transferred to nitrocellulose membranes according to the method of Towbin et al. (33). The immunoblotting was performed as described previously (28). Autoradiographs were quantitated using a personal densitometer (Molecular Dynamics Inc., Sunnyvale, CA).

**Steady State Surface Distribution**—The steady state surface distribution of wt and mutant MR constructs was measured by binding of iodinated antibodies and by biotinylation with equal results. The first method was as described in Schweizer et al. (23) except that the anti-CD-MPR antibodies were iodinated by ANAWA Trading SA (Zürich, Switzerland) according to their standard protocol. For the biotinylation, confluent cells in 12-well plates were incubated for 24 h with 100 µg each of pepstatin A and leupeptin. The cells were washed twice with ice-cold PBS-supplemented with 0.25 mM MgSO4 and 0.7 mM CaCl2 (PBS++) before incubation with either PBS++ (surface) or PBS++ containing 0.05% saponin (total). The cells were then incubated for 15 min on ice with either PBS++ containing 3 mg/ml sulfo-NHS-biotin (surface) or with PBS++ containing 3 mg/ml sulfo-NHS-biotin and 0.05% saponin (total). Subsequently the cells were first washed once with PBS++ (0% control) and then with PBS++ containing 50 mM glycine/0.05% saponin (total) and then twice with either PBS++ (surface) or PBS++ supplemented with 0.05% saponin (total). Finally, the cells were solubilized in 100 mM phosphate buffer, pH 8, containing 1% Triton X-100, transferred to a 1.5-ml tube, and centrifuged for 20 min at 70,000 rpm in a TLA100.3 rotor (Beckman Instruments). Immunoprecipitation and Western blots of the chimeras was performed as mentioned (28). The biotinylated fraction of the proteins was then detected by enhanced chemiluminescence using streptavidin- horseradish peroxidase.

**Internalization Assay**—Cells grown in 6-well plates were rinsed twice with ice-cold PBS++ and then incubated with 1.5 ml of 1 mg/ml sulfo-NHS-biotin in PBS++ for 15 min to biotinylate surface proteins. Biotinylation was stopped by washing once with 50 mM glycine in PBS++ and twice with PBS++. Some of the cells were then incubated at 37 °C with prewarmed growth medium containing 10% fetal calf serum and 20 mM Hepes, pH 7.4, for different periods of time (1, 2, and 3 min). The cells were returned to 4 °C to stop internalization and then incubated on ice twice for 20 min in a freshly prepared glutathione solution (50 mM glutathione, 75 mM sodium chloride, 1 mM EDTA, pH 8) to remove the biotin from proteins that were present on the cell surface. In addition, two samples that were not incubated at 37 °C were treated either with 0% control or without (100% control) the glutathione solution. After reduction, the excess glutathione was quenched with a 5-min incubation in PBS++ containing 5 mg/ml iodoacetamide. The cells were then washed once with PBS++, lysed in 1 ml of 100 mM sodium phosphate buffer, pH 8, containing 1% Triton X-100, and harvested with a 25-gauge needle connected to a 1-ml syringe. The resulting homogenates were centrifuged for 20 min at 70,000 rpm in a TLA100.3 rotor, and the supernatant was subjected to immunoprecipitation and Western blotting as described (28). The biotinylated fraction of the proteins was then detected by enhanced chemiluminescence using streptavidin-horseradish peroxidase.

**Assays and Miscellaneous Methods**—β-Hexosaminidase activity was
The amino acids of the cytoplasmic tail are shown in single letter code. For the CD-MPR, 49 amino acids of the total 67 amino acids of the cytoplasmic tail are shown. The half boxes represent the termini of the single transmembrane domain (TMD) of each receptor. The diaromatic motif that is present in all three cytoplasmic tails at about the same distance from the transmembrane domain is highlighted in boldface type. Two sequences are shown for the MR (human (h) and mouse (m)) and one sequence for the CD-MPR, because it is identical in all known species.

determined as described (28). Protein concentration was determined with the Bio-Rad protein assay kit by using protein standard I.

RESULTS

The Human MR Contains a Pair of Di-aromatic Amino Acids at the Same Position as the Di-aromatic Endosomal Sorting Motif of the CD-MPR—In a previous study, it was demonstrated that proper sorting of the CD-MPR in endosomes depends on a pair of aromatic amino acids (Phe-Trp) at positions 18 and 19 from the transmembrane domain (23). The residues Phe<sup>18</sup>-Trp<sup>19</sup> are required to keep CD-MPRs from being delivered to lysosomes where they would be degraded. Because the replacement of the Phe<sup>18</sup>-Trp<sup>19</sup> sequence with other aromatic residues did not alter the intracellular distribution of the receptor, it appears that the important feature for endosomal sorting of the CD-MPR is the presence of two aromatic residues in its cytoplasmic tail. In our studies to define features of the human MR required for its sorting in endosomal compartments, we initially compared the amino acid sequences of the cytoplasmic tails of the human and mouse MRs and the CD-MPR, for which the cytosolic tail is identical in all known species (Fig. 1). The evaluation revealed that both MRs contain a di-aromatic amino acid sequence (YF) in their cystolic tail. As in the CD-MPR, the YF motif of the human MR is located at positions 18 and 19 from the transmembrane domain, whereas this distance is increased by only one amino acid in the mouse MR. In addition, both MRs share a phenylalanine five residues upstream from the di-aromatic sequence with the CD-MPR. This Phe<sup>13</sup> in the CD-MPR is part of a signal (F<sup>13</sup>XXX<sup>18</sup>F<sup>19</sup>, with F<sup>18</sup> being the key residue) for rapid internalization from the plasma membrane (34). A similar signal (FXNPXY) is also required for the internalization of the low density lipoprotein receptor (35).

Taken together, Tyr<sup>18</sup> and Phe<sup>19</sup> are likely candidates to direct sorting of the MR in endosomes. In analogy to the CD-MPR and the low density lipoprotein receptor, Tyr<sup>18</sup> could also be essential for internalization from the plasma membrane.

**MFR-MRMR YF-AA/YQTL and MFR-MRMR YF-AA/YSAF Are Missorted to Lysosomes—**To test if the di-aromatic amino acid sequence Tyr<sup>18</sup>-Phe<sup>19</sup> indeed functions to avoid delivery of the MR to dense lysosomes, we created a variety of chimeric constructs that contain the cytoplasmic and transmembrane domains of the MR linked to the luminal domain of the CD-MPR (Fig 2). The cytosolic tail and transmembrane anchor have previously been shown to be sufficient for correct intracellular trafficking of the MR (18). The CD-MPR luminal domain, on the other hand, while being an ideal reporter molecule to study lysosomal avoidance/delivery, does not contain endosomal sorting information (28, 36).

The first chimeric construct includes a wt MR cytosolic tail sequence (MPR-MRMR wt, Fig. 2), whereas the second chimera has Tyr<sup>18</sup> and Phe<sup>19</sup> in the cytoplasmic domain changed to alanines (MPR-MRMR YF-AA, Fig. 2). Because, as discussed above, Tyr<sup>18</sup> might be part of a potential signal for rapid endocytosis, we considered the possibility that mutation of Tyr<sup>18</sup>-Phe<sup>19</sup> also affects internalization of the MR. This would prevent a correct assessment of the intracellular sorting step in endosomes. Two additional constructs were therefore prepared in which the tetrapeptide sequences YQTL and YSAF were attached to the C terminus of the chimeric constructs (MPR-MRMR wt, Fig. 2). Both motifs are classical signals for rapid internalization from the plasma membrane with the essential features of a tyrosine in the first position and a bulky hydrophobic residue in the last position. The first signal, YQTL, combines attributes of the internalization motifs of Lamp1 (YQTI) (37, 38) and TGN38 (YQRL) (39, 40), whereas the second signal, YSAF, corresponds to the internalization motif of the mouse polymeric immunoglobulin receptor (41). The signals YQTL and YSAF were chosen to facilitate internalization from the plasma membrane without affecting intracellular sorting. As a control, these sequences were also attached to the wt construct (MPR-MRMR YQTL and MPR-MRMR YSAF, Fig. 2).

The six constructs were stably transfected into mouse L cells and several clones of each mutant were selected for subsequent experiments. The intracellular distribution of the expressed proteins was then analyzed by isosmotic Percoll density gradients, which separate dense lysosomes from other organelles (28, 42). To this end, cell lines were first preincubated for 24 h in medium supplemented with pepstatin A and leupeptin to inhibit degradation of receptors that had entered lysosomes. The cells were then harvested, homogenized with a ball-bearing homogenizer, and subjected to 17.5% Percoll density gradient fractionation. The fractions from the gradients were analyzed in three pools: pool I (bottom of the gradient) contains the bulk of the dense lysosomes (70–80% of β-hexosaminidase activity), whereas low density membranes including endosomes, the Golgi complex, plasma membrane, and the endosomal reticulum are recovered in pool III (top of gradient) (28, 42). Intermediate density membranes are found in pool II. The distribution of the various constructs was determined by electrophoresis of the Percoll density fractions followed by Western blotting with anti-CD MPR monoclonal antibodies. As shown in Fig. 3, 20 ± 3% of the wild type chimeric receptor MPR-MRMR wt accumulated in dense lysosomes. A similar accumulation in lysosomes was found when Tyr<sup>18</sup> and Phe<sup>19</sup> were replaced with alanines (MPR-MRMR YF-AA, 18 ± 9%). However, the same mutant receptor was found to be highly missorted to lysosomes.
when the internalization signals YQTL or YSAF were attached to its C terminus. 46 ± 5% of MPR-MRMR YF-AA/YQTL and 47 ± 8% of MPR-MRMR YF-AA/YSAF were recovered in dense lysosomes (pool I). In contrast, the addition of the internalization motifs to the wild type MPR-MRMR did not significantly influence lysosomal accumulation (MPR-MRMR YQTL, 26 ± 8% and MPR-MRMR YSAF, 19 ± 10%).

These results suggest that the Tyr18-Phe19 motif in the cytoplasmic tail of the MR mediates endosomal sorting. The importance of Tyr18-Phe19 to prevent trafficking to lysosomes, however, is only apparent after the attachment of an internalization motif to the mutant receptor. This suggests that, as expected, the YF-AA mutation also impairs internalization at the cell surface. Thus a greater fraction of MPR-MRMR YF-AA is likely to be present at the plasma membrane and therefore not available for mislocalization to lysosomes.

The Di-aromatic Motif Tyr18-Phe19 in the Cytosolic Tail of the MR Mediates Endosomal Sorting—Given the potential importance of Tyr18-Phe19 for both endosomal sorting and internalization from the plasma membrane, we next analyzed the intracellular distribution of the mutant receptors in more detail.

First, we determined the fraction of the various chimeric molecules present at the cell surface at steady state by measuring the binding of 125I-labeled anti-CD-MPR antibody to intact cells (surface receptor) versus binding to cells permeabilized with 0.1% saponin (total receptor) and by biotinylation of intact cells (surface receptor) versus biotinylation of cells permeabilized with saponin (total receptor). As shown in Table I, MPR-MRMR YF-AA is greatly enriched at the plasma membrane, 79 ± 11% of this mutant receptor was present on the cell surface compared with 46 ± 13% for the MPR-MRMR wt. The addition of the internalization sequences YQTL or YSAF reduced cell surface expression of MPR-MRMR YF-AA almost to wild type level (MPR-MRMR YF-AA/YQTL, 57 ± 11% and MPR-MRMR YF-AA/YSAF, 53 ± 24%). The presence of an additional endocytosis signal in the MPR-MRMR wt decreased the fraction of mutant receptors at the cell surface to 39 ± 12% (MPR-MRMR YQTL) and 25 ± 3% (MPR-MRMR YSAF), respectively. These data confirm that mutation of Tyr18-Phe19 in the cytoplasmic tail of the MR significantly impairs internalization from the plasma membrane.

Depending on the fraction of mutant receptors at the plasma membrane at steady state, the amount of internal receptors available for mislocalization to lysosomes differs. To take this skew distribution into account, we next recalculated the accumulation of the mutant receptors in lysosomes as a percentage of the internal pool of receptors that is in fact available for endosomal sorting or mislocalization to lysosomes, respectively. As shown in Table I, 37% of the intracellular wild type chimeric receptor (MPR-MRMR wt) was recovered in the lysosomal fraction. MPR-MRMR YQTL and MPR-MRMR YSAF were found to accumulate in dense lysosomes to a similar extent than MPR-MRMR wt (43 and 25%, respectively). In contrast, the intracellular pools of all three mutant receptors with the YF-AA mutation were entirely missorted to lysosomes. 100% of the intracellular MPR-MRMR YF-AA/YSAF were recovered in the dense lysosomal fraction, and comparable accumulations were obtained for MPR-MRMR YF-AA/YQTL (107%) and MPR-MRMR YF-AA (87%). Taken together, these results convincingly demonstrate that the di-aromatic amino acid motif in the cytosolic tail of the MR mediates endosomal sorting.

Tyr18 Is a Key Residue for Both Endosomal Sorting and

### Table I

| Construct                  | % of surface<sup>a</sup> | Gradient in lysosomes<sup>b</sup> | % of internal pool in lysosomes<sup>c</sup> |
|---------------------------|--------------------------|----------------------------------|--------------------------------------------|
| MPR-MRMR wt               | 44 ± 14                  | 20 ± 3                           | 36                                         |
| MPR-MRMR YF-AA            | 79 ± 11                  | 18 ± 9                           | 87                                         |
| MPR-MRMR YF-AA/YQTL       | 57 ± 11                  | 46 ± 5                           | 107                                        |
| MPR-MRMR YF-AA/YSAF       | 53 ± 24                  | 47 ± 8                           | 100                                        |
| MPR-MRMR YQTL             | 39 ± 12                  | 26 ± 8                           | 43                                         |
| MPR-MRMR YSAF             | 25 ± 3                   | 19 ± 10                          | 25                                         |
| MPR-MRMR Y18A             | 68 ± 13                  | 25 ± 3                           | 78                                         |
| MPR-MRMR F19A             | 49 ± 10                  | 25 ± 7                           | 49                                         |

<sup>a</sup> Percentage of the various mutant receptors that were present at the cell surface at steady state. The values are expressed as mean ± S.E. from three to eight separate experiments.

<sup>b</sup> Percentage of the various mutant receptors recovered in dense lysosomes on Percoll density gradients. The values are expressed as the mean ± S.E. from three to eight separate experiments.

<sup>c</sup> The percentage of the internal pool of mutant receptors that is in lysosomes was calculated using the numbers for the percentage on the surface and in lysosomes.
Constructs were prepared that have Tyr 18 and Phe19 individual of the signal. In addition, as expected, Tyr18 but not Phe19 ble I). These results indicate that Tyr 18 is the key residue for MPR-MRMR Y18A than of MPR-MRMR F19A is mislocalized to lysosomes (78% surface (68%) than MPR-MRMR F19A (49%), which showed a MPR-MRMR Y18A was present to a larger extend at the cell surface (55%) than MPR-MRMR F19A (25%) (Table I). However, the subcellular distribution is given in Table I and Fig. 4. On Percoll density gradients both mutant receptors were recovered in dense lysosomes to the same degree (MPR-MRMR Y18A, 25% and MPR-MRMR F19A, 25%) (Table I). However, MPR-MRMR Y18A was present to a larger extent at the cell surface (68%) than MPR-MRMR F19A (49%), which showed a similar accumulation as MPR-MRMR wt (Table I). Therefore a much greater fraction of the internal pool of MPR-MRMR Y18A than of MPR-MRMR F19A is mislocalized to lysosomes (78% for MPR-MRMR Y18A versus 49% for MPR-MRMR F19A) (Table I). These results indicate that Tyr 18 is the key residue for endosomal sorting with Phe19 serving as a secondary component of the signal. In addition, as expected, Tyr18 but not Phe19 appears to be crucial for internalization.

To corroborate the latter finding, the initial rates of internalization of MPR-MRMR wt, MPR-MRMR YF-AA, MPR-MRMR Y18A, and MPR-MRMR F19A were determined. To do this, cells stably expressing the different constructs were grown in 12-well plates, chilled on ice, and incubated with sulfo-NHS-SS-biotin to label cell surface proteins. The cells were then warmed to 37 °C for various times (up to 3 min) to allow for protein internalization and were subsequently chilled on ice again to stop any further membrane trafficking. Glutathione, a nonmembrane permeable reducing agent, was then added to the medium to strip the biotin from proteins remaining at the cell surface. After the glutathione treatment, the cells were solubilized and the various receptors were immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with streptavidin-horseradish peroxidase to detect biotinylated proteins. The quantification of multiple experiments is shown in Fig. 4. Both MPR-MRMR wt and MPR-MRMR F19A were rapidly internalized. In contrast, MPR-MRMR YF-AA and MPR-MRMR Y18A showed very slow rates of internalization. Taken together, these findings demonstrate that Tyr18 is essential for both endosomal sorting and internalization.

DISCUSSION

The results presented in this paper demonstrate that the di-aromatic motif Tyr18-Phe19 in the cytosolic tail of the MR is functional for endosomal sorting. These two aromatic residues were sufficient to prevent lysosomal delivery of the majority of a chimeric molecule consisting of the transmembrane and cytosolic domain of the MR fused to the luminal domain of the CD-MPR. Replacement of the aromatic residues with alanines on the other hand led to a complete mislocalization of the internal pool of chimeras to lysosomes.

The Tyr18-Phe19 motif of the MR is the second example of a di-aromatic amino acid sequence in the cytoplasmic tail of a receptor that has now been shown to mediate endosomal sorting. Previously, the di-aromatic motif Phe18-Trp19 was found to be both necessary and sufficient for sorting of the CD-MPR in endosomes (23). Replacing Phe18-Trp19 with other aromatic amino acids including Tyr-Phe did not impair endosomal sorting indicating that two aromatic residues are the essential feature for this sorting step. Whereas Trp19 was identified as the primary component of the CD-MPR di-aromatic motif, Tyr18 was shown to be the key residue of the MR signal. Strikingly, both Tyr18-Phe19 of the human MR and Phe18-Trp19 of the CD-MPR are located in the cytosolic tail at the same distance from the transmembrane domain, whereas this distance is increased by only one amino acid in the mouse MR. This is particularly intriguing because alterations in the spacing of the di-aromatic amino acid sequence of the CD-MPR relative to the transmembrane domain impaired endosomal sorting. Both the addition of five residues and removal of four residues upstream of the Phe18-Trp19 sequence caused mislocalization of the receptor to lysosomes. Spacing of the sorting signal has also been found to be critical for efficient sorting of the lysosomal membrane protein Lamp1 to lysosomes (43). In addition to the MR and the CD-MPR, the receptor for phospholipase A2 also contains two aromatic residues that are located at approximately the same distance from the transmembrane domain (Tyr17-Tyr18) and thus are likely to function in the endosomal sorting of the protein. Taken together, it appears that a di-aromatic amino acid motif at the correct position from the transmembrane domain proves to be a general determinant for endosomal sorting.

Alanine substitution of Tyr18 did not only affect endosomal sorting of the MR-CD-MPR chimeras but also led to a very slow rate of internalization from the plasma membrane, consistent with the presence of overlapping signals for internalization and endosomal sorting in the MR cytosolic tail. Further evidence for a dual function comes from the analysis of the mutants MPR-MRMR YF-AA/YQTL and MPR-MRMR YF-AA/YSAF. Attachment of a typical endocytosis signal at the C terminus rescued the internalization defect of the mutants, whereas all the internal molecules were still missorted to lysosomes. In addition, a role for Tyr18 of the MR in endocytosis has previously been shown by Kruskal et al. (18) in chimeric receptors containing the ectodomain of the Fc receptor and the transmembrane and cytoplasmic domains of the MR. A more complete MR internalization sequence can be predicted by a comparison with other receptors known to be internalized from the plasma membrane. Previous studies have identified a tyrosine-based internalization signal for the low density lipoprotein receptor that consists of the amino acid sequence FXXNPXY (35). A similar sequence, FXXFXXX, has been shown for the CD-MPR to mediate rapid internalization from the plasma membrane (34). In analogy to the signals of the low density lipoprotein receptor and the
CD-MPR, it seems likely that the internalization motif of the MR consists of the sequence 13FENTLY18, which encompasses Tyr18 of the di-aromatic endosomal sorting motif. As suggested for the MR, the signals for internalization (13FPPPAPGR) and endosomal sorting (Pho18-Trp19) of the CD-MPR overlap as well.

Consistent with the identification of an active sorting step that prevents lysosomal delivery of the MR, newly synthesized MR exhibits a half-life of 33 h, indicating that each molecule of receptor recycles many times between endosomes and the plasma membrane before degradation (7). An equally long half-life (>40 h) was found for the CD-MPR that, as discussed above, avoids transport to lysosomes via the same motif as the MR. In this regard, it is interesting to note that for both the MR and the CD-MPR the transmembrane domain and cytosolic tail have been shown to be sufficient for their normal trafficking, whereas the two luminal domains do not appear to contain any significant sorting information (18, 28). The similarity in the trafficking signals between the MR and the CD-MPR is also reflected in their overlapping subcellular distributions. In a study by Messner (31) the MR was shown to be highly enriched in liver membrane fractions immuno-isolated with antibodies to the CD-MPR, whereas a severalfold reduced enrichment was found in fractions obtained with antibodies to the cation-indepent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR/IGFII receptor). Whereas both MRs cycle between TGN, endosomes and the plasma membrane to transport newly synthesized acid hydrolases to lysosomes; subcellular fractionation and electron microscopy studies demonstrated that the distribution of the two receptors along the recycling route is not identical (31, 44, 45). More of the CI-MPR/IGFII receptor than the CD-MPR was found in late endosomes, and in particular, the CD-MPR was shown to be enriched in tubulovesicular structures associated with endosomes (44). These structures are believed to mediate receptor recycling from endosomal compartments (44). It remains to be shown if this specific distribution of the CD-MPR is also true for the MR.

The endosomal sorting motif that we have identified in the cytosolic domain of the MR may well function in the recruitment of the receptor into specialized subregions of the endosomes, which give rise to vesicles leaving the organelle as part of the recycling pathway. As established for signals mediating sorting in the TGN and at the plasma membrane (46–48), the di-aromatic sorting motif could thereby interact with coat proteins that are involved in vesicle formation on endosomes. When the sorting motif is mutated, the receptor would migrate to other subregions within the endosomes that either fuse directly with lysosomes or alternatively give rise to vesicles that fuse with these organelles. With respect to the endosomal sorting of the mannose 6-phosphate receptors, Diaz and Pfeffer (49) have recently identified a novel protein (Tip47) that selectively binds to the cytosolic tails of both receptors and was reported to play a role in their endosome to TGN transport. Interestingly, mutation of Phe18,Trp19 in the cytosolic tail of the CD-MPR strongly reduced binding of recombinant Tip47 to a CD-MPR cytoplasmic tail glutathione S-transferase fusion protein, indicating that the interaction of the two proteins depends on the di-aromatic motif. The CI-MPR/IGFII receptor, however, lacks such a di-aromatic motif in its cytoplasmic domain. In addition, mutation of both the single tryptophan at position 42 and three aromatic/hydrophobic residues Tyr24, Tyr26 and Val29 at a position similar to Phe18,Trp19 had no effect on binding (49, 50). Because Tyr24, Tyr26 and Trp42 are the only aromatic residues in the part of the receptor important for Tip47 binding, these results indicate that aromatic amino acids are not important for the binding of Tip47 to the CI-MPR/IGFII receptor. Futhermore, Orsel et al. (50) identified the sequence 49PPAPGR as a putative binding site for Tip47. It will therefore be of particular interest to determine whether Tip47 binds to the Tyr18-Phe19 motif in the MR.

In conclusion, we have shown in the present paper that the cytoplasmic tail of the MR contains a sorting motif that serves to prevent trafficking to lysosomes and facilitates transport out of endosomes. The results of this study can now be used to determine whether endosomal sorting is important for the biological function of the MR, in particular phagocytosis and signaling.

Acknowledgment—We thank Dr. D. Messner for kindly providing antibodies against the CD-MPR.

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