

Tail-specific Antibodies That Block Return of 46,000 *M_r* Mannose 6-Phosphate Receptor to the *trans*-Golgi Network

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Abstract. Recycling of 46,000 *M_r* mannose 6-phosphate receptor (MPR 46) was investigated by microinjection of F_{ab} fragments against small epitopes within the cytoplasmic domain of the receptor. F_{ab} fragments against the peptide 43–47 (Ala-Tyr-Arg-Gly-Val) efficiently blocked return of MPR 46 to the TGN. Antibody-induced redistribution resulted in accumulation of MPR 46 within an endosomal compartment, from which it recycled to the plasma membrane. Rab5 and rab7, markers for early and late endosomes, respectively, were not detectable in the compartment of redistributed MPR 46, suggesting that it represents

a specialized endosomal subcompartment. The bulk of redistributed MPR 46 did not colocalize with endocytosed fluid-phase marker, suggesting that it accumulates at a site where MPR 46 has been segregated from endocytosed material, which is destined for transport to lysosomes. Peptide 43–47 contains a tyrosine (residue 44) which has been shown earlier to be part of an internalization signal for MPR 46 (Johnson, K. F., W. Chan, and S. Kornfeld. 1990. *Proc. Natl. Acad. Sci. USA.* 87:10010–10014). The role of tyrosine residue 44 as part of a putative multifunctional sorting signal is discussed.

MPR 46 (46,000 *M_r*, mannose 6-phosphate receptor)¹ is one of the two receptors that are involved in sorting and transport of newly synthesized lysosomal enzymes (for review see Kornfeld and Mellman, 1989). 46,000 *M_r* MPR (MPR 46) is thought to bind lysosomal enzymes in the TGN for segregation from resident Golgi proteins and from proteins of the secretory pathway. After transfer to the endosomal compartment, receptor–ligand complexes dissociate due to the acidic pH. Lysosomal enzymes reach their final destination by an unknown mechanism, whereas MPR 46 recycles back to the TGN. While sorting of newly synthesized lysosomal enzymes towards lysosomes is carried out by both, MPR 46 and the 300,000 *M_r* MPR (MPR 300), only MPR 46 mediates export of a portion of the lysosomal enzymes (Chao et al., 1990). On the other hand, binding of lysosomal enzymes at the cell surface and receptor-mediated endocytosis is a unique function of MPR 300 (Stein et al., 1987). Nevertheless, MPR 46 is transported to the plasma membrane and recycles between plasma membrane and endosomes.

The functional diversity of the two MPRs is reflected by their overlapping but not identical subcellular distribution. By immunoelectron microscopy, both receptors were found in the TGN and in early as well as late endosomes (Bleekemolen et al., 1988; Matovcik et al., 1990). In the en-

dosomal compartment, however, partial segregation of the two receptors has been observed (Klumperman et al., 1993). MPR 300 was found to be enriched in the large vacuolar part of endosomes, when compared with associated tubular extensions and small vesicles surrounding the endosomes (referred to as ATV). The ratio of immunogold-labeled MPR 46 over MPR 300 was higher in ATV than in the vacuolar part of the endosomes, suggesting differential sorting of the two receptors.

In most instances, sorting of receptors is regulated by signals in their cytoplasmic domain, which are recognized by cytosolic transport factors. Though recently it has been postulated that also the extracellular or transmembrane domains play a role for recycling of MPR 300, the signals involved have not been identified to date (Dintzis and Pfeffer, 1990). The sorting of receptors into clathrin-coated pits of the plasma membrane depends on a tyrosine residue, or an aromatic amino acid as part of an internalization signal in their cytoplasmic domain. This signal is recognized by HA-2 adaptors, the protein complex which induces the assembly of the clathrin coat around receptors at the plasma membrane (for review see Pearse and Robinson, 1990). The tyrosine-containing internalization signal is thought to be exposed in a β -turn conformation within the cytoplasmic domain as shown for low density lipoprotein (LDL) receptor, transferrin receptor, and lysosomal acid phosphatase (Bansal and Gierasch, 1991; Collawn et al., 1990; Ktistakis et al., 1990; Eberle et al., 1991). For the rapid internalization of MPR 46, a more complex signal has been characterized by

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1. Abbreviation used in this paper: MPR, mannose-6 phosphate receptor.

site-directed mutagenesis. Johnson et al. (1990) suggested two distinct internalization signals in the cytoplasmic domain of bovine MPR 46, the first signal including amino acids Phe-12 and Phe-17, and the second including Tyr-44. For the rapid endocytosis of human MPR 46 the last seven amino acids of the cytoplasmic domain proved to be essential (Weber et al., in preparation).

A second clathrin-dependent pathway is involved in transport from the TGN to the endosomal compartment. HA-1 adaptors, which are thought to mediate assembly of coated vesicles at the TGN, have been shown to bind to an as yet unidentified signal in the cytoplasmic domain of MPR 300 and MPR 46 (Glickman et al., 1989; Sosa et al., 1993). Additional signals that regulate exit from the endosomal compartment and recycling of receptors to the TGN or to the plasma membrane, and putative cytosolic proteins involved in these sorting steps remain to be characterized.

We have used peptide-specific antibodies that recognize small peptide epitopes within the cytoplasmic domain of human MPR 46 to screen for sorting signals involved in its intracellular transport. In the present study we show that F_{ab} fragments against peptide 38–52 of the cytoplasmic domain of MPR 46, when microinjected into human fibroblasts, prevent recycling of MPR 46 to the TGN, resulting in accumulation of MPR 46 in a vesicular compartment that is related to endosomes. Antibodies against the peptide Ala-Tyr-Arg-Gly-Val, which constitutes the central epitope of peptide 38–52, were most efficient in causing this redistribution. Antibodies against peptide 7–21 did not alter the distribution of MPR 46.

Materials and Methods

Cells

Human fibroblasts were obtained from a skin biopsy for diagnostic purpose and cultured as previously described (Sly and Grubb, 1979). BHK 21 cells overexpressing human MPR 46 were cultured as previously described (Wendland et al., 1991).

Peptide-specific Antibodies and F_{ab} Fragments

Antisera against synthetic peptides which correspond to amino acids 7–21 (peptide A) and 38–52 (peptide B) of the cytoplasmic domain of human MPR 46 (starting with Arg 212 as amino acid no. 1) have been described earlier (Nadimpalli et al., 1991).

Affinity purification of antibodies was performed with peptides that had been coupled to Affigel 10 (Bio-Rad, München, Germany) as described (Nadimpalli et al., 1991; Geuze et al., 1984). To subfractionate antibodies against peptide B, sequential chromatography of the antiserum was performed on immobilized decapeptides as outlined in Fig. 1. For each decapeptide, rechromatography was performed to deplete all antibodies that were specific for the immobilized peptide before the antiserum was loaded to the subsequent peptide column.

F_{ab} fragments of affinity-purified antibodies were generated by digestion with papain at a ratio of 1 mg papain per 50 mg of antibody for 30 min at 37°C as previously described (Hille et al., 1992).

Immunoprecipitation of Radioiodinated MPR 46

Immunoprecipitation of ^{125}I -MPR 46 was performed essentially as previously described (Nadimpalli et al., 1991). For competition experiments, 30 μ g of affinity-purified antibodies were preincubated with 300 μ g of decapeptide in 100 μ l PBS containing 0.05% Triton X-100 for 1 h at room temperature. After adding 10 ng of ^{125}I -MPR 46, the samples were incubated for 30 min at room temperature and overnight at 4°C. The immune complexes were adsorbed to Pansorbin (60 μ l of a 10% suspension) (Calbiochem, Frankfurt a. M., Germany) for 1 h at 4°C, washed as described, solubilized,

and subjected to SDS-PAGE under reducing conditions (Laemmli, 1970). After detection by fluorography, the bands corresponding to MPR 46 were cut from the gel to quantitate ^{125}I -radioactivity by scintillation counting.

Microinjection and Internalization of Endosomal Markers

Cells were grown for 2 d on polylysine-coated coverslips. Microinjection was performed as previously described (Hille et al., 1992). After an incubation for 0.5–3.0 h at 37°C, the cells were either fixed immediately for immunofluorescence or loaded with endosomal markers.

Internalization of Antibodies Against MPR 46. Cells were preincubated for 2 \times 30 min at 37°C with bicarbonate-free MEM containing 10 mM Hepes-NaOH, pH 7.4 (referred to as MEM-Hepes). The anti-MPR 46 mAb 10C6 (A. Hille-Rehfeld, unpublished data) was offered at 0.2 mg/ml in MEM-Hepes for 10 min at 37°C. Cells were either washed immediately with ice-cold PBS and fixed for immunofluorescence, or washed twice with PBS (37°C), incubated for 20 min at 37°C in MEM-Hepes, and then fixed. Where indicated, cycloheximide (final concentration 280 μ M) was added to the culture medium 60 min before starting the microinjection, and all subsequent incubations were carried out in the presence of 280 μ M cycloheximide.

Internalization of Transferrin. Transferrin was saturated with iron as described (Klausner et al., 1983). Cells were preincubated for 2 \times 30 min in MEM-Hepes, and transferrin was offered to the cells at 1 mg/ml in MEM-Hepes for 30 min at 37°C. Internalization was stopped by rapid cooling on ice followed by two washes with ice-cold PBS and fixation for immunofluorescence.

Internalization of HRP. Cells were washed three times with PBS (37°C) and HRP (10 mg/ml in MEM-Hepes) (grade II; Boehringer, Mannheim, Germany) was offered at 37°C. Internalization was stopped by rapid cooling on ice and three washes with ice-cold PBS. Cells were then incubated three times for 5 min with ice-cold 5% FCS in PBS, followed by three washes with ice-cold PBS and fixation for immunofluorescence.

Indirect Immunofluorescence

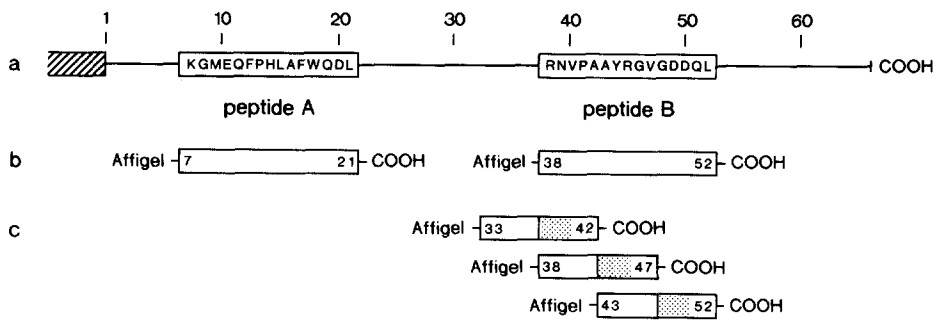
Immunofluorescence was performed as described (Hille et al., 1992) using the following marker antibodies: MPR 46 was either labeled with the mouse mAb 10C6 or a polyclonal IgG raised in goat and purified using immobilized protein G (Wenk et al., 1991). MPR 300 was labeled with a polyclonal IgG raised in goat and likewise purified using immobilized protein G (Causin et al., 1988). The TGN was labeled with the mAb 100/3 directed against the γ -subunit of HA-1 adaptors (Ahle et al., 1988). Lysosomes were labeled with a polyclonal antiserum against cathepsin D raised in goat (Hasilik and Neufeld, 1980). Early or late endosomes were labeled with affinity-purified rabbit antibodies against rab5 or rab7, respectively (Chavrier et al., 1990). Internalized transferrin or HRP were detected with rabbit anti-human transferrin (Janssen Biochemica, Beerse, Belgium) or goat anti-HRP antibodies (Sigma, Deisenhofen, Germany), respectively. Fluorochrome-labeled antibodies (Dianova, Hamburg, Germany) were preabsorbed against serum proteins from other species. Their specificity was confirmed, and cross-reactivity of primary or secondary antibodies was ruled out by control experiments with various combinations of antibodies. Injected Ig or F_{ab} fragments were detected with donkey anti-rabbit Ig conjugated with AMCA; compartment-specific marker antibodies were detected with donkey anti-mouse Ig conjugated with Texas red or donkey anti-goat Ig conjugated with FITC. For simultaneous detection of injected F_{ab} fragments from rabbit and marker Ig raised in rabbit, we used goat anti-rabbit Ig conjugated with AMCA and goat anti-rabbit Fc conjugated with Texas red.

Conventional epifluorescence was performed with an Axioplan microscope (Zeiss, Oberkochen, Germany) using the following filter combinations: G 365, FT 395, BP 450–490 for detection of AMCA; BP 450–490, FT 510, LP 520 for detection of FITC; and BP 530–585, FT 600, LP 615 for detection of Texas red. For photographic documentation, confocal laser scanning microscopy (LSM; Zeiss) was performed as described (Hille et al., 1992). Stacks of confocal optical sections (\sim 0.8 μ m thick) were used for serial overlay by digital image processing.

Results

Purification and Characterization of Peptide-specific Antibodies

Polyclonal rabbit antibodies that recognize 15-mer peptides



within the cytoplasmic tail of MPR 46, corresponding to amino acids 7–21 or 38–52 (Fig. 1 *a*), were described earlier (Nadimpalli et al., 1991). These antibodies have been shown to be monospecific for MPR 46 by immunoprecipitation of the receptor from cell lysates. In addition, immunoprecipitation of radioiodinated MPR 46 was competed by the synthetic peptides used for immunization, but not by unrelated peptides. For microinjection experiments, affinity-purified antibodies were prepared by affinity chromatography, using immobilized peptides 7–21 or 38–52 (Fig. 1 *b*).

To obtain antibodies against smaller epitopes within peptide 38–52, an antiserum against the 15-mer peptide was subfractionated by sequential affinity chromatography on three overlapping decapeptides (amino acids 33–42, 38–47, and 43–52) that had been coupled to Affigel-10 with their NH₂-terminal amino acid (Fig. 1 *c*). The immobilized decapeptides had been designed in such a way that, starting from the NH₂-terminal end of peptide 38–52, antibodies were consecutively bound to the five amino acids that are exposed at the COOH terminus of each immobilized decapeptide. The five additional amino acids at the NH₂-terminal side of the immobilized decapeptides served as a spacer arm, to minimize steric hindrance for binding of antibodies. The resulting fractions of antibodies are referred to as anti-38–42, anti-43–47 and anti-48–52, according to the corresponding pentapeptide epitopes. The two latter antibody fractions may recognize, in addition, epitopes that are constituted by the NH₂-terminal boundaries of the pentapeptides (amino acids 42/43 and 47/48). The affinity-purified antibodies were shown to recognize native MPR 46 by immunoprecipitation of radioiodinated receptor (shown for immunoprecipitation with antibodies anti-43–47 in Fig. 2, third lane). The peptide specificity of the antibody fractions was proven by immunoprecipitation of radioiodinated MPR 46 in the presence or absence of a 1,000-fold molar excess of free decapeptides. As shown in Fig. 2 for antibodies anti-43–47, the decapeptide 38–47, which had been used for affinity purification, efficiently competed immunoprecipitation (fourth lane). Competition was also observed with decapeptide 43–52 (fifth lane), which overlaps the peptide used for affinity purification, exposing the peptide 43–47 at its NH₂-terminal end. Decapeptides that correspond to amino acids adjoining the NH₂- or COOH-terminal end of peptide 43–47 did not compete (sixth and seventh lanes).

Similar results were obtained for the antibodies anti-38–42 and anti-48–52 (not shown). These results demonstrate the peptide-specificity of the antibody fractions which were obtained by affinity chromatography.

Redistribution of MPR 46 by Microinjection of Peptide-specific Antibodies Against Amino Acids 38–52 of Its Cytoplasmic Tail

F_{ab} fragments that recognize the 15-mer peptides 7–21 or 38–52 were injected into human skin fibroblasts, and cells were incubated for up to 3 h at 37°C to allow binding of the injected antibodies to the cytoplasmic domain of MPR 46. The cells were then fixed, permeabilized, and processed for immunofluorescence to compare the steady state distribution of MPR 46 in control cells and injected cells. Injected cells were identified by coinjected FITC-BSA (Fig. 3, *c* and *d*). To visualize the distribution of MPR 46, a mouse mAb (10C6), which recognizes the luminal domain of MPR 46 and does not interfere with binding of the injected antibodies, was used in combination with Texas red-conjugated anti-mouse Ig as a second antibody (Fig. 3, *a* and *b*). In non-injected cells (cells on the left in Fig. 3, *a* and *b*), the highest

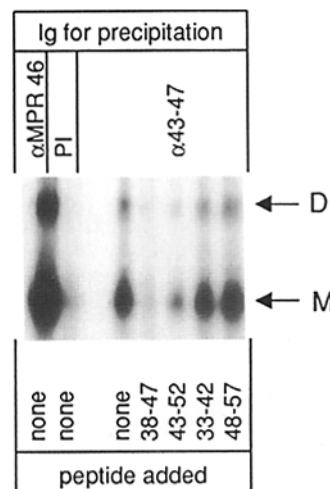


Figure 2. Peptide-specific immunoprecipitation of MPR 46. Immunoprecipitation of radioiodinated MPR 46 was performed with affinity-purified Ig against whole MPR 46 (α MPR 46), preimmune Ig (PI), or affinity-purified Ig against peptide 43–47 (α 43–47), which corresponds to part of the cytoplasmic domain of MPR 46 (compare Fig. 1). Where indicated, antibodies were preincubated with an excess of synthetic peptide. Immunoprecipitates were analyzed by SDS-PAGE, and MPR 46 was detected by fluorography. *M*, monomeric MPR 46; *D*, SDS-resistant dimer of MPR 46.

anti 7-21

anti 38-52

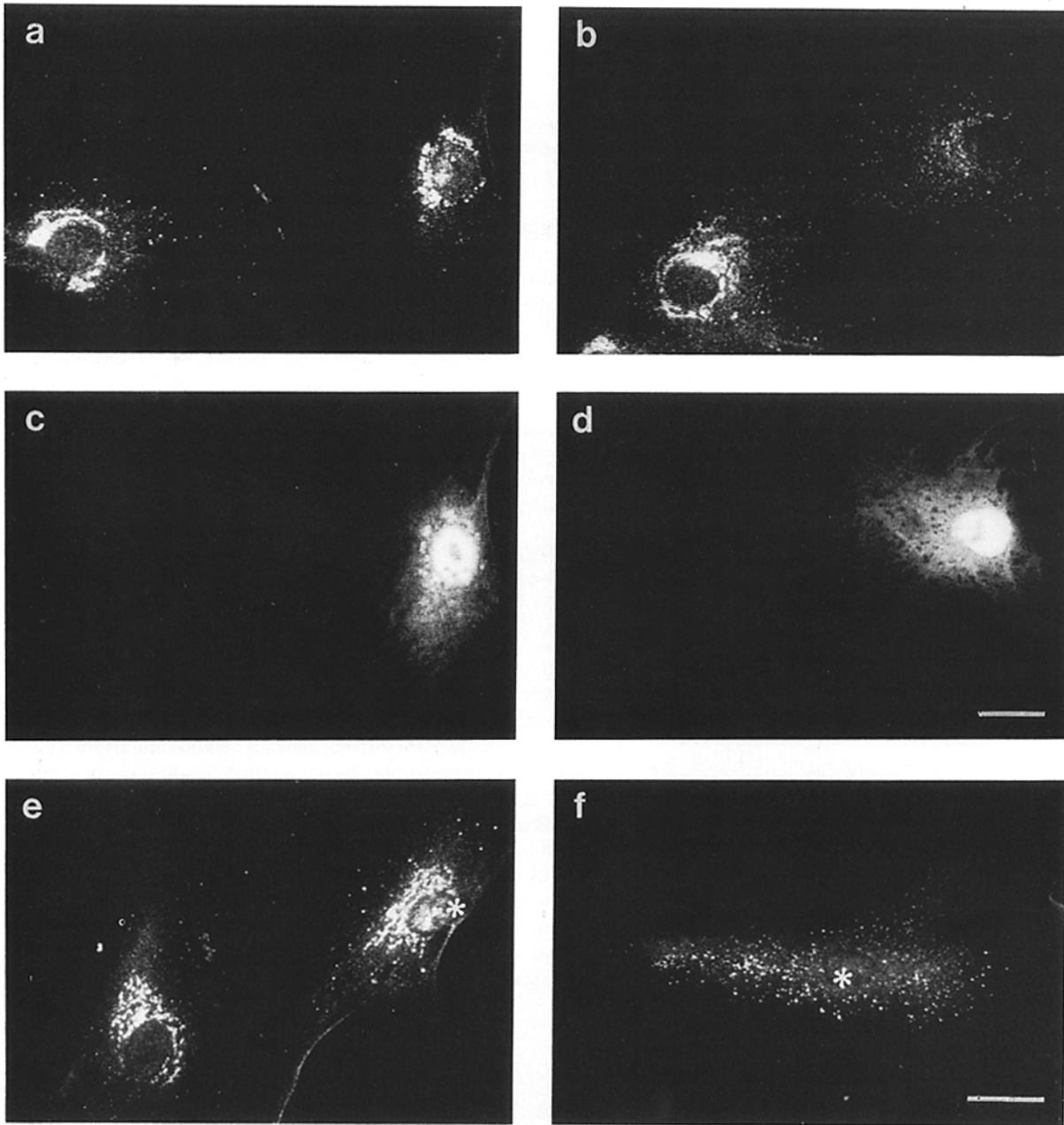


Figure 3. Redistribution of MPR 46 by microinjected F_{ab} fragments against peptide 38-52. Human fibroblasts were microinjected with F_{ab} fragments (9 mg/ml) against peptide 7-21 (left panels) or against peptide 38-52 (right panels). 30 min (a-d) or 120 min (e and f) after injection, cells were fixed and processed for immunofluorescence detection of MPR 46 (a, b, e, and f). Coinjected FITC-BSA (0.5 mg/ml) is shown in c and d. a and b show the same sector as c and d, respectively. Asterisks in e and f indicate injected cells that had been identified by immunofluorescence staining of injected antibodies. Bars, 20 μ m.

labeling for MPR 46 was found in perinuclear, tubular structures which colocalized with HA-1 adaptors as a marker for the TGN (see below, Fig. 5, a and b). In addition, MPR 46 was found in vesicular structures in the periphery of non-injected cells, which partially colocalized with endocytosed transferrin (not shown). Microinjection of F_{ab} fragments against peptide 38-52 caused redistribution of MPR 46 from the TGN to vesicular structures that were scattered throughout the cytoplasm (cell on the right in Fig. 3 b). Already 30 min after injection, the TGN was almost devoid of MPR 46

(Fig. 3 b) and the effect was stable for at least 3 h (shown for 2 h, Fig. 3 f). After microinjection of F_{ab} fragments against peptide 7-21, the immunofluorescence pattern of MPR 46 was not changed, neither 30 min after microinjection (Fig. 3 a) nor at later time points (up to 3 h after microinjection, Fig. 3 e). For both peptides, injection of Ig provided results that were indistinguishable from those described above for injection of F_{ab} fragments (compare Fig. 4 a for injection of anti 38-52 Ig). Injection of preimmune rabbit IgG or injection of buffer alone did not change the im-

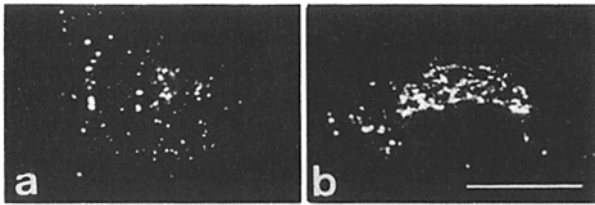


Figure 4. Coinjected antigenic peptide prevents antibody-induced redistribution of MPR 46. Human fibroblasts were microinjected with antibodies against peptide 38–52 (1 mg/ml) together with peptide 7–21 (*a*) or peptide 38–52 (*b*) (10 mg/ml each). 90 min after injection, cells were fixed and processed for immunofluorescence detection of MPR 46. Only injected cells are shown, which were identified by immunofluorescence detection of injected Ig. Bar, 20 μ m.

munofluorescence pattern of MPR 46, except for a slight and fully reversible distortion of the TGN at \sim 5 min after microinjection (not shown).

The fact that antibodies against peptide 7–21 did not change the distribution of MPR 46 raised the question whether the epitope 7–21 is accessible in situ for binding of injected antibodies to the cytoplasmic domain of MPR 46. We therefore investigated whether injected antibodies were bound to membranes containing MPR 46. In human fibroblasts, which express relatively low amounts of endogenous MPR 46, it was difficult to detect membrane-bound injected antibodies because the cytosol was filled with excess unbound antibodies. In transfected Ltk-cells, which overexpress human MPR 46, Ig against peptides 7–21 and 38–52 were both bound to membranes which contain MPR 46 but, as in fibroblasts, redistribution was observed only with Ig against peptide 38–52 (not shown).

Redistribution of MPR 46 by Ig against peptide 38–52 was completely abolished by coinjected peptide 38–52 at about 1,300-fold molar excess (Fig. 4 *b*), but not by the same concentration of the irrelevant peptide 7–21 (Fig. 4 *a*). This result confirms that antibody-induced redistribution of MPR

46 was a result of specific binding of antibodies to the epitope 38–52 within the cytoplasmic domain of MPR 46.

We next sought to find out which part of peptide 38–52 was responsible for antibody-induced redistribution of MPR 46. F_{ab} fragments against the three peptides 38–42, 43–47, or 48–52 were microinjected, and redistribution of MPR 46 was monitored by comparing the immunofluorescence pattern of MPR 46 (Fig. 5, *upper panel*) with that of HA-1 adaptors (Fig. 5, *lower panel*) as a marker for the TGN (Ahle et al., 1988; Geuze et al., 1991). F_{ab} fragments against the peptide 38–42 did not change the immunofluorescence pattern of MPR 46. As in non-injected control cells, MPR 46 was found in perinuclear tubular structures, which were also labeled for HA-1 adaptors, and in peripheral endosomal vesicles (Fig. 5, *a–d*). F_{ab} fragments against the central peptide 43–47 efficiently caused redistribution of MPR 46 from the TGN into vesicular structures which were scattered throughout the cytoplasm (Fig. 5, *e and f*), and which were indistinguishable from those obtained upon microinjection of antibodies against the 15-mer peptide 38–52. Microinjection of F_{ab} fragments against the COOH-terminal peptide 48–52 also caused the disappearance of MPR 46 from the TGN, but the vesicular staining for MPR 46 was less pronounced compared with cells that had been microinjected with F_{ab} fragments against peptide 43–47 (Fig. 5, *g and h*). For all three peptides, similar results were obtained with injected Ig (not shown). We therefore can exclude that the failure of F_{ab} fragments against peptide 38–42 to induce redistribution of MPR 46 resulted from proteolytic cleavage of the Ig. The immunofluorescence pattern of HA-1 adaptors (Fig. 5, *lower panel*) and galactosyltransferase (not shown) was not altered by any of the microinjected antibodies, suggesting that the structure of the TGN had not been disturbed.

Recent reports that the tyrosine residue within peptide 43–47 is relevant for endocytosis of bovine MPR 46 which was overexpressed in mouse L-cells (Johnson et al., 1990) raised the question whether redistribution of MPR 46 by antibodies against peptide 43–47 may result in arrest of the

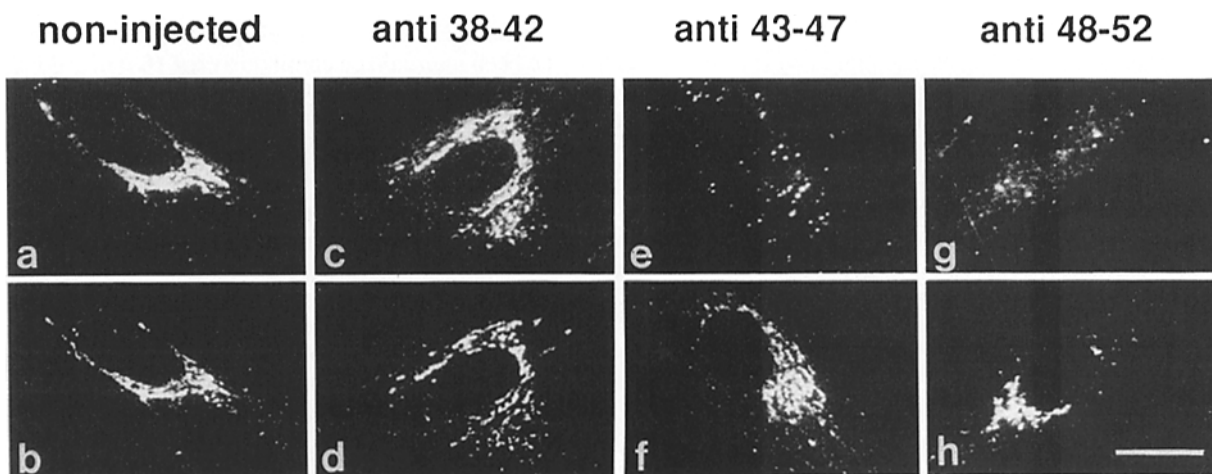


Figure 5. Redistribution of MPR 46 by microinjection of F_{ab} fragments against peptides 43–47 and 48–52. Human fibroblasts were microinjected with antibodies against peptides 38–42 (7.6 mg/ml), 43–47 (9 mg/ml), or 48–52 (9 mg/ml). 90 min after injection, cells were fixed and processed for simultaneous immunofluorescence detection of MPR 46 (*upper panels*) and γ -adaptin (*lower panels*). Injected cells were identified by detection of injected F_{ab} fragments. Pairs of vertically aligned panels show double staining of one cell. Bar, 20 μ m.

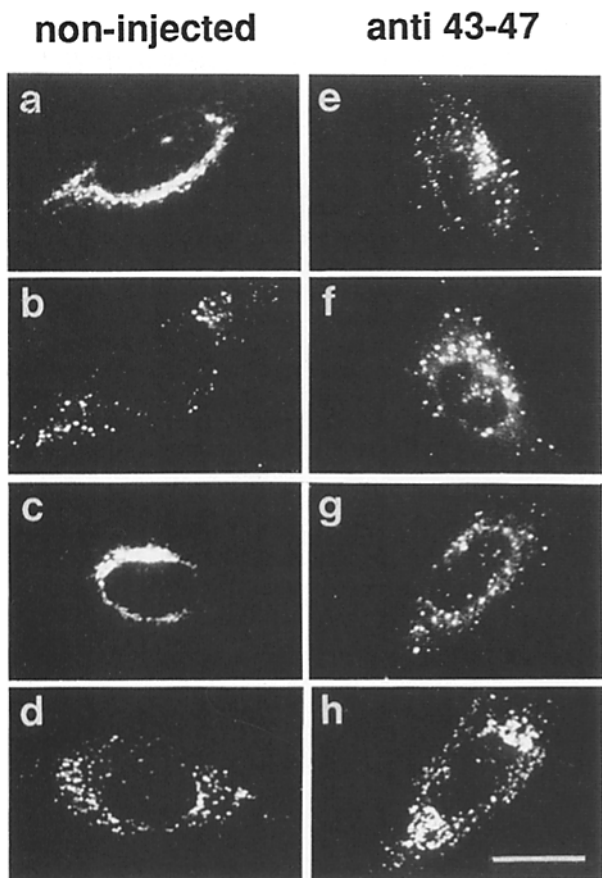


Figure 6. Injected F_{ab} fragments do not cause missorting of MPR 46 to lysosomes. Human fibroblasts were microinjected with antibodies against peptide 43–47 (4.5 mg/ml). 90 min after injection, cells were fixed and processed for simultaneous immunofluorescence detection of MPR 46 (a, c, e, and g) and cathepsin D (b, d, f, and h). Injected cells were identified by detection of injected F_{ab} fragments. Cells shown in c, d, g, and h were treated with leupeptin and pepstatin (final concentration 100 μ M each) starting from 17 h before microinjection and throughout until cells were fixed. Pairs of vertically aligned cells (a/b, c/d, e/f, and g/h) show double staining of one cell. Bar, 20 μ m.

receptor at the plasma membrane. We therefore investigated the cell surface pattern of MPR 46 after microinjection of F_{ab} fragments against peptide 43–47 by immunolabeling of cells which had not been permeabilized. The cell surface staining of nonpermeabilized cells showed a faint, diffuse immunolabeling, which was similar in injected and control cells (not shown). The dots containing redistributed MPR 46 were accessible to immunolabeling by the monoclonal antibody against the luminal domain only after permeabilization of cells. These data suggest that the bulk of redistributed MPR 46 is contained in an intracellular compartment.

The observed redistribution of MPR 46 may reflect arrested trafficking of MPR 46, resulting in accumulation of the receptor within a compartment that is part of its normal recycling pathway. Alternatively, it may reflect missorting of the receptor to lysosomes due to tagging of the cytoplasmic domain with F_{ab} fragments. To investigate this issue, the immunofluorescence pattern of MPR 46 was compared with that of the lysosomal enzyme cathepsin D. In non-injected cells (Fig. 6, a and b) there was essentially no overlap in the

distribution of MPR 46 (a) and cathepsin D (b), as was expected according to the recycling pathway of MPR 46 (reviewed by Kornfeld and Mellman, 1989). Occasionally, few vesicular structures were found to be labeled for both MPR 46 and cathepsin D, which may be explained by the fact that newly synthesized cathepsin D, like any lysosomal antigen, must pass through the endosomal compartment on its way to lysosomes. In cells injected with F_{ab} fragments against peptides 43–47 or 48–52, there was no evidence for increased colocalization of MPR 46 and cathepsin D (shown for injection of anti-peptide 43–47 in Fig. 6, e and f). To exclude a missorting of redistributed MPR 46 to lysosomes and rapid degradation therein, cells were treated with the protease inhibitors leupeptin and pepstatin, which have been shown earlier to prevent degradation of a mutant form of MPR 46 which is missorted to lysosomes (Peters et al., 1990). Also in cells that had been injected in the presence of protease inhibitors, there was no substantial colocalization of redistributed MPR 46 (Fig. 6 g) with cathepsin D (Fig. 6 h), suggesting that redistributed MPR 46 is neither missorted to nor degraded within lysosomes.

Redistributed MPR 46 Accumulates in an Endosomal Subcompartment

To investigate whether redistributed MPR 46 resides in an endosomal compartment, cells were loaded with the mAb 10C6 against the extracellular or ecto-domain of MPR 46, which is specifically imported by MPR 46 that recycles between cell surface and endosomes (see Stein et al., 1987). These studies were carried out with BHK-cells overexpressing human MPR 46 (BHK-MPR 46 cells), because cell surface expression of MPR 46 in human fibroblasts was too low to detect import of anti-MPR 46 antibodies. Injection of F_{ab} fragments against peptide 43–47 into BHK-MPR 46 cells resulted in redistribution of MPR 46 from the perinuclear region (Fig. 7 a) to small vesicles scattered throughout the cytoplasm, indicating that also in these cells the microinjected antibodies block transport of MPR 46 to the perinuclear region (shown in Fig. 7 c for a cell that had been fixed and processed for immunofluorescence 70 min after microinjection).

In non-injected BHK-MPR 46 cells, ectodomain antibodies that had been internalized continuously for 10 min at 37°C (Fig. 7 b) colocalized with MPR 46 (Fig. 7 a) in peripheral endosomal vesicles, but not with MPR 46 in perinuclear, Golgi-like structures. In contrast, redistributed MPR 46 in injected cells (Fig. 7 c) did not colocalize with ectodomain antibodies that had been internalized for 10 min (Fig. 7 d). Only after 30 min of continuous internalization, partial colocalization of internalized ectodomain antibodies and redistributed MPR 46 was observed (not shown). When ectodomain antibodies were offered to the cells for 10 min followed by a chase of 20 min, the great majority of the structures containing redistributed MPR 46 (Fig. 7 g) were labeled with endocytosed ectodomain antibodies (Fig. 7 h).

To investigate whether loading of endosomes with endocytosed anti-MPR 46 antibodies in injected cells indicates recycling of redistributed MPR 46 between the endosomal compartment and the plasma membrane, or whether internalization depended on newly synthesized MPR 46, protein synthesis was blocked by incubation with cycloheximide. Treatment of cells with 280 μ M cycloheximide reduced

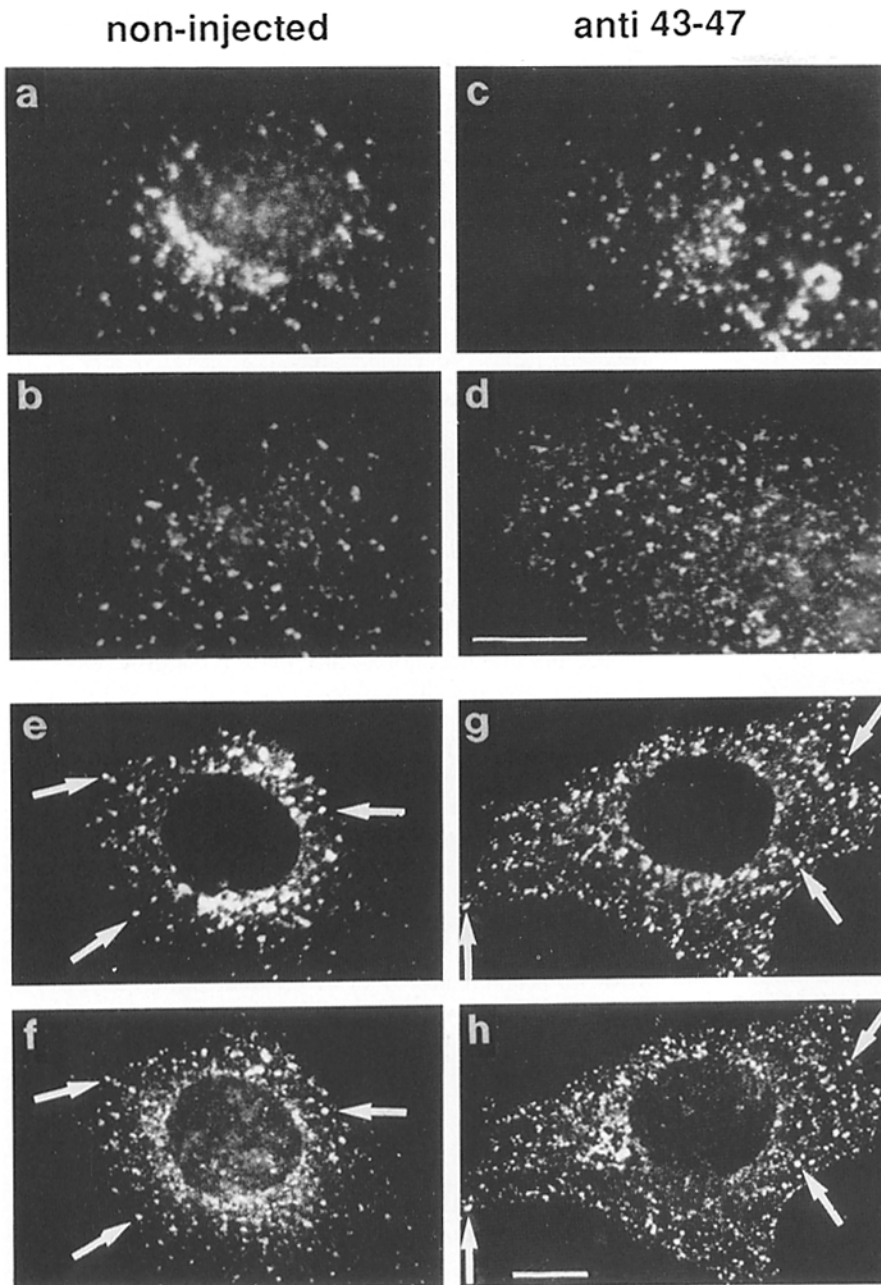


Figure 7. The compartment of redistributed MPR 46 is accessible to endocytosed antibodies. BHK cells overexpressing human MPR 46 were microinjected with F_{ab} fragments against peptide 43–47 (4.5 mg/ml). 60 min after injection, cells were incubated for 10 min with a mouse mAb against the extracellular domain of MPR 46 and fixed either immediately (*a–d*) or after a chase of 20 min (*e–h*). Cells were then permeabilized for simultaneous immunofluorescence staining of total cellular MPR 46 with goat antibody (*a*, *c*, *e*, and *g*) and internalized mouse anti-MPR 46 antibody (*b*, *d*, *f*, and *h*). Injected cells were identified by detection of injected rabbit F_{ab} fragment. Pairs of vertically aligned panels (*alb*, *cld*, *elf*, and *gh*) show double staining of one cell. Arrows point to some of the structures that show labeling for both endocytosed anti-MPR 46 and total cellular MPR 46. Bars, 10 μ m.

[35 S]methionine incorporation into newly synthesized proteins to $\sim 10\%$ of the control value within 30 min, and to $\sim 5\%$ within 60 min (not shown). To make sure that MPR 46 which had been synthesized before addition of cycloheximide had left the biosynthetic pathway (see Hille et al., 1990), cells were incubated with 280 μ M cycloheximide for a total of 2 h before anti-MPR 46 antibodies were offered for endocytosis. Also after treatment with cycloheximide, endocytosed anti-MPR 46 antibodies colocalized with total cellular MPR 46 both in non-injected and injected cells (not shown). This indicates that redistributed MPR 46 is not immobilized but can recycle to the plasma membrane.

Colocalization of endocytosed ectodomain antibodies with redistributed MPR 46 was also observed at 20°C, i.e., under conditions of arrested transport from early to late endosomes (Marsh et al., 1983). After 10 min of internaliza-

tion at 20°C, a 50-min chase was necessary to obtain a similar degree of colocalization as after a 20 min chase at 37°C (not shown). Since incubation at reduced temperature results in transient arrest rather than irreversible blockade of transport, it was important to control the efficiency of the arrest under the conditions used. When receptor-mediated endocytosis of arylsulfatase A was followed at 20°C in BHK-cells, transport to dense lysosomes did not occur for at least 75 min during a continuous incubation with arylsulfatase A-containing medium, whereas at 37°C about 45% of endocytosed arylsulfatase A was found in the dense lysosomal fraction (R. Bresciani and K. von Figura, unpublished observations). These data suggest efficient arrest within the endocytic pathway at 20°C. Therefore, colocalization of redistributed MPR 46 with ectodomain antibodies internalized at 20°C suggests that the pathway of MPR 46 from the cell surface to the com-

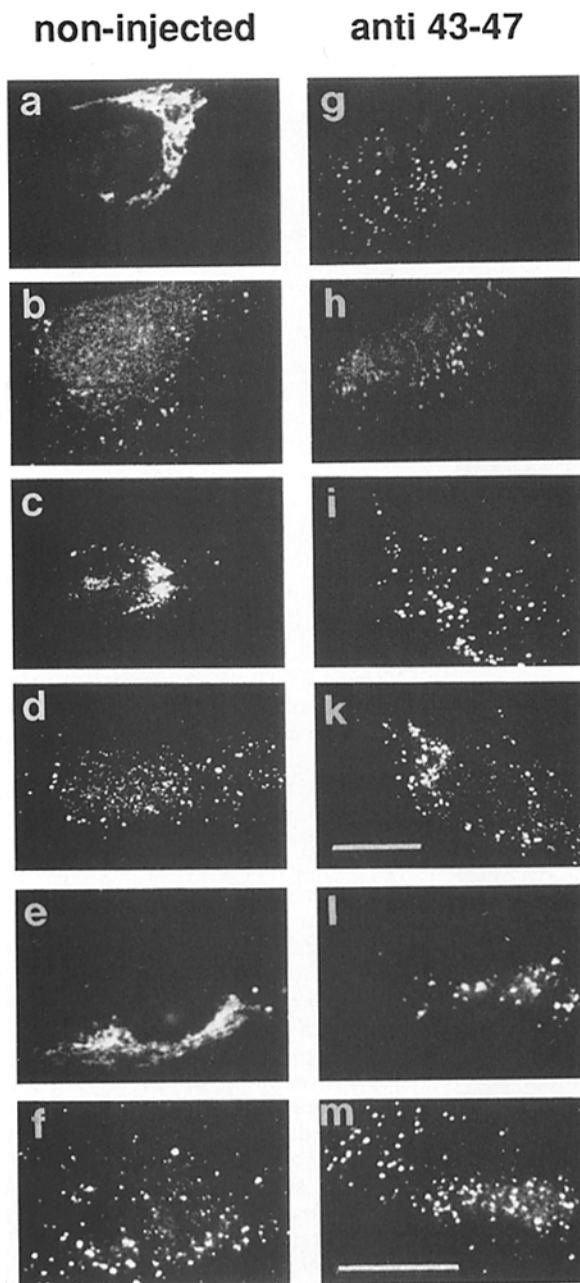


Figure 8. Redistributed MPR 46 does not colocalize with the endosomal markers rab5, rab7, or HRP. Human fibroblasts were microinjected with F_{ab} fragments against peptide 43–47 (9 mg/ml). 90 min after injection, cells were either fixed immediately or loaded for 30 min with HRP and then fixed. Cells were then processed for simultaneous immunofluorescence detection of MPR 46 (a, c, e, g, i, and l), and either rab5 (b and h), rab7 (d and k), or HRP (f and m). Injected cells were identified by detection of injected F_{ab} fragments. Pairs of vertically aligned panels (a/b, c/d, e/f, g/h, i/k, and l/m) show double staining of one cell. Note that e, f, l, and m are shown with higher magnification than the upper panels a–d and g–k. Bars, 20 μm

partment of redistributed MPR 46 does not pass beyond the site of the 20°C block.

To further characterize the compartment of redistributed MPR 46, double immunofluorescence staining was performed with various endosomal markers. Rab5 (Fig. 8, b and

h) and rab7 (Fig. 8, d and k), which bind to the cytoplasmic face of early and late endosomes, respectively (Chavrier et al., 1990), showed a vesicular pattern which is typical for endosomes. In non-injected control cells, rab5 (Fig. 8 b) occasionally colocalized with MPR 46 (Fig. 8 a) in peripheral vesicular structures as shown by overlay of confocal images (not visible in Fig. 8), whereas rab7 (Fig. 8 d) was not detectable in vesicular structures containing MPR 46 (Fig. 8 c). Conversely, MPR 300 partially colocalized with rab7, but essentially not with rab5 (not shown). When the immunofluorescence pattern of redistributed MPR 46 in microinjected cells (Fig. 8, g and i) was compared with that of rab5 (Fig. 8 h) or rab7 (Fig. 8 k), colocalization was found rarely. Endocytosed transferrin, another marker for early endosomes (Schmid et al., 1988 and references cited therein), also showed partial colocalization with MPR 46 in non-injected cells, but very little if any with redistributed MPR 46 in injected cells (not shown). MPR 300 (Fig. 9 b) showed a high degree of colocalization with MPR 46 in non-injected cells or cells injected with control antibodies (Fig. 9 a), which was especially evident in the perinuclear region. After microinjection of F_{ab} fragments against peptides 43–47 or 48–52, the immunofluorescence pattern of MPR 300 (Fig. 9, d and f) was not changed and, in contrast to control cells, did not overlap with that of redistributed MPR 46 (Fig. 9, c and e). Endocytosed HRP as a marker for fluid-phase uptake has been shown to label the endocytic route all along the way to lysosomes (for review see Helenius et al., 1983). When microinjected cells were loaded for 15, 30, or 60 min with HRP (shown for the 30-min time point in Fig. 8, f and m), there was little colocalization with MPR 46 both in control and injected cells (Fig. 8, e and l, respectively).

Taken together, these results suggest that redistributed MPR 46 accumulates in an as yet unidentified endosomal subcompartment which is accessible to endocytosed antibodies but is separate from the general endocytic route which leads to the lysosome.

Discussion

Antibody-induced Redistribution of MPR 46 to an Endosomal Subcompartment

MPR 46 recycles between TGN and endosomes for sorting of newly synthesized lysosomal enzymes. In addition, MPR 46 recycles between endosomes and plasma membrane (Duncan and Kornfeld, 1988; Stein et al., 1987). Immunofluorescence staining showed that in human fibroblasts the major portion of MPR 46 is localized in the TGN, suggesting that exit from the TGN where newly synthesized lysosomal enzymes are bound is the rate limiting step in transport of MPR 46, whereas return from endosomes occurs rapidly in these cells.

Microinjection of F_{ab} fragments against the peptide 43–47 resulted in rapid and efficient redistribution of MPR 46 from the TGN to vesicular structures that were scattered throughout the cytoplasm. The use of monovalent F_{ab} fragments precluded possible artifacts due to antibody-induced cross-linking. Moreover, redistributed receptor did not colocalize with cathepsin D, indicating that tagging with the F_{ab} fragments did not induce missorting to lysosomes. Redistributed MPR 46 mediated import of antibodies against

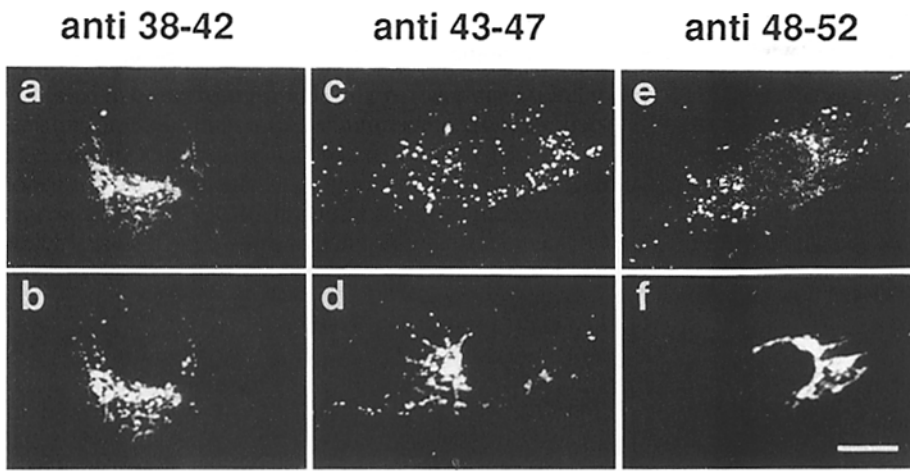


Figure 9. Antibody-induced redistribution is specific for MPR 46. Human fibroblasts were microinjected with F_{ab} fragments against peptides 38–42 (a and b), 43–47 (c and d) or 48–52 (e and f). For concentrations of injected F_{ab} see Fig. 5. 90 min after injection, cells were fixed and processed for simultaneous immunofluorescence detection of MPR 46 (upper panels) and MPR 300 (lower panels). Injected cells were identified by detection of injected F_{ab} fragments. Pairs of vertically aligned panels show double staining of one cell. Bar, 20 μ m.

its extracytoplasmic domain under conditions of blocked protein synthesis. This result suggests that MPR 46 was not immobilized by the bound antibodies, and recycling between endosomes and the plasma membrane was not blocked.

Antibody-induced redistribution was selectively observed for MPR 46, the immunofluorescence pattern of markers for TGN, endosomes or lysosomes being unchanged. Remarkably, the intracellular distribution of MPR 300, which contains a sequence motif similar to peptide 43–47 (Tyr-X-X-Val, see Oshima et al., 1988), was not changed in microinjected cells, suggesting that the peptide-specific antibodies did not cross react with epitopes that are relevant for transport of MPR 300.

The compartment of redistributed MPR 46 did not contain HA-1 adaptors or galactosyltransferase as markers of the TGN or Golgi cisternae (Geuze et al., 1991; Nilsson et al., 1993), whereas essentially all the structures containing redistributed MPR 46 were accessible to endocytosed ectodomain antibodies. These results suggest that redistributed MPR 46 resides in a compartment which is connected to the endosomal pathway. Lack of colocalization of redistributed MPR 46 with a fluid-phase marker and with markers for early or late endosomes suggests that the compartment of redistributed MPR 46 is separate from the main route of endocytic traffic which leads to lysosomes. In this context, it is interesting that recent results obtained by immunoelectron microscopy have shown the presence of MPR 46 in tubular extensions of both early and late endosomes, which do not contain asialoglycoprotein receptor, and which are only rarely stained by fluid-phase markers (Klumperman et al., 1993). These data suggest that within endosomes, MPR 46 is segregated into membranes which exclude material destined for return to the plasma membrane (asialoglycoprotein receptor) or for transport to lysosomes (fluid-phase marker) and which may be involved in segregation of MPR 46 for return to the TGN. The data presented here are compatible with the view that the compartment of redistributed MPR 46 is related to or derived from the above mentioned tubular extensions of endosomes seen in immunoelectron microscopy. The compartment of redistributed receptor may therefore constitute an intermediate compartment which is involved in segregation of MPR 46 from the general endocytic route, which leads to lysosomes. This compartment is located proximal to the 20°C block within endosomes and must be

passed by MPR 46 before it returns to the TGN. To characterize the ultrastructure of the compartment of redistributed MPR 46 by immunoelectron microscopy, and to investigate its molecular composition as well as its function is the aim of our future studies. The elucidation of its putative function in sorting and vesicular transport of MPR 46 will depend on the production of antibodies that specifically bind to this compartment.

Sorting Events Involved in Recycling of MPR 46 from Endosomal Membranes

Antibody-induced redistribution of MPR 46 can be explained by blockade of a single transport step. Blockade of transport either from endosomal membranes to the TGN, or within the endosomal compartment are both sufficient to shift the main localization of MPR 46 from the TGN to endosomal membranes. Since microinjected F_{ab} fragments anti-43–47 inhibit return of MPR 46 to the TGN, binding of F_{ab} fragments to peptide 43–47 in the cytoplasmic domain of MPR 46 most likely masks information contained within this epitope, which is required for return of MPR 46 to the TGN. The sorting step which is affected by F_{ab} fragments anti-43–47 may reside either directly at the exit site from endosomal membranes or at an earlier transport step between different membranes of the endosomal pathway. From our data, there is no evidence that recycling to the plasma membrane is affected by binding of F_{ab} fragments anti-43–47.

The effect of blocking antibodies can be explained either by competition with binding of cytosolic transport factors to the cytoplasmic domain of MPR 46, or by interference with changes in the conformation or quaternary structure of MPR 46 that may be essential for its recycling to the TGN. If the epitope of the blocking antibodies is part of a signal for binding of cytosolic transport factors, then the peptide may be expected to interact with cytosolic transport factors and compete with their binding to the cytoplasmic domain of MPR 46, and therefore cause a similar redistribution of MPR 46 as was observed after injection of peptide-specific antibodies. In our hands, peptide 38–52, when injected into fibroblasts at a concentration of 10 mg/ml did not cause redistribution of MPR 46 (not shown), whereas antibody-induced redistribution of MPR 46 was prevented by coinjecting the peptide at the same concentration. There are two feasible ex-

planations for the failure to induce redistribution of MPR 46 by injection of the peptide alone. First, the affinity of the synthetic peptide to cytosolic transport factors may be too low to compete with receptors *in situ*, if the conformation of the 15-mer peptide in solution differs from that of the corresponding peptide within the cytoplasmic domain of MPR 46. Alternatively, the peptide may be rapidly degraded by cytosolic proteases when injected alone without being complexed by coinjected Ig. It will be interesting to investigate whether peptides containing the epitope 43–47 are capable of binding cytosolic proteins, or proteins associated with the cytoplasmic face of endosomal membranes or transport vesicles, using an *in vitro* assay.

Is Tyrosine 44 Part of a Multifunctional Signal Involved in Sorting at the Plasma Membrane and Intracellular Traffic?

Recent results obtained by *in vitro* mutagenesis of aromatic amino acids in the cytoplasmic domain of MPR 46 suggested that amino acid residue Tyr-44 is part of a signal for efficient endocytosis of MPR 46 (Johnson et al., 1990). Moreover, the peptide 43–47 (Ala-Tyr-Arg-Gly-Val) displays some homology to peptide sequences that were shown to bind plasma membrane coated vesicle adaptors (reviewed by Pearse and Robinson, 1990). Binding of antibodies against peptide 43–47 to the cytoplasmic domain of MPR 46 may therefore be expected to interfere with binding of plasma membrane adaptors and with clathrin-mediated endocytosis of MPR 46. It was, therefore, surprising that antibodies against peptide 43–47 interfered with an intracellular sorting step. These apparently conflicting results may be explained by the assumption that the epitope surrounding Tyr-44 is part of a multifunctional signal, which binds several different transport factors involved in endocytosis or in intracellular sorting. Such a multifunctional signal can be compared to a master key which opens different locks. The binding of cytosolic transport factors to their specific target membranes must then be determined either by a third component that is present in or associated with the target membrane, or by conformational changes in the cytoplasmic domain of the receptor. The latter may modulate the signal depending on the membrane in which the receptor resides. The assumption that one signal may be involved in several sorting events is supported by recent reports that the tyrosine-containing signal for endocytosis is also involved in polar sorting of several receptors and lysosomal acid phosphatase from the TGN to the basolateral plasma membrane in epithelial cells (Brewer and Roth, 1991; Casanova et al., 1991; Hunziker et al., 1991; Prill et al., 1993). If Tyr-44 of MPR 46 is part of a multifunctional sorting signal involved both in endocytosis and intracellular sorting, one should observe antibody-induced accumulation of MPR 46 both in endosomes and at the plasma membrane. Since binding of antibodies to the putative internalization signal may cause a reduction in the rate of internalization rather than a blockade, as was the case after mutagenesis of the signal (Johnson et al., 1990), the resulting increase of the steady state concentration of MPR 46 at the plasma membrane might be too low to be detected by immunofluorescence. Similarly, if tagging of MPR 46 by microinjected F_{ab} fragments induces internalization, the steady state concentration of MPR 46 at the plasma membrane may increase little, if at all. Alternatively, the failure

to detect antibody-induced accumulation of MPR 46 at the plasma membrane may be explained by the assumption that plasma membrane adaptors have a higher affinity for the multifunctional signal than putative transport factors binding to the signal at the endosome. Sorting at the endosome thus would be more sensitive for interference by microinjected antibodies.

Conclusion

Our results show that microinjection of peptide-specific antibodies and immunocytochemistry can be used to identify epitopes within the cytoplasmic domain of MPR 46 which are relevant for its intracellular transport. To further characterize the signals and putative cytosolic transport factors involved in exit of MPR 46 from the endosomal pathway, complementary biochemical and cell biological studies need to be done. So far, our data suggest that return from endosomes to the TGN is not a bulk flow process, but requires specific sorting signals.

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