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The carboxy-terminal peptides of 46 kDa and 300 kDa mannose 6-phosphate receptors share partial sequence homology and contain information for sorting in the early endosomal pathway

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SUMMARY

Recycling of mannose 6-phosphate receptors was investigated by microinjection of F_{ab} fragments against their carboxy-terminal peptides (residues 54-67 or 150-164 of the cytoplasmic domain of 46 kDa and 300 kDa mannose 6-phosphate receptor, respectively). For each receptor, masking the carboxy-terminal peptide by the corresponding F_{ab} fragments resulted in complete depletion of the intracellular pool. Redistributed 300 kDa mannose 6-phosphate receptor was shown to accumulate at the plasma membrane and to internalize anti-ectodomain antibodies. Internalization of anti-ectodomain antibodies was also observed for redistributed 46 kDa mannose 6-phosphate receptor. Semiquantitative analysis suggested that for both redistributed receptors the amount of intracellularly accumulated anti-ectodomain antibodies was reduced. In addition, downstream transport along the endosomal pathway was slowed down. These data suggest that sorting information for early steps in the endocytic pathway is contained within the carboxy-terminal peptides of mannose 6-phosphate receptors.

Key words: Mannose 6-phosphate receptor, Internalization, Endosomal sorting, Peptide-specific antibody, Microinjection

INTRODUCTION

Mannose 6-phosphate receptors (MPR) recycle between *trans*-Golgi network (TGN) and endosomes for transport of newly synthesized lysosomal enzymes. Both the 46 kDa and 300 kDa MPR (referred to as MPR 46 and MPR 300, respectively) also recycle between plasma membrane and endosomes, but only MPR 300 has been found to internalize exogenous ligands. Endogenous and exogenous ligands dissociate in the endosomal pathway for further transport to lysosomes, whereas MPRs recycle either to the TGN or to the plasma membrane and, as a consequence, are not detectable in lysosomes (for review see Hille-Rehfeld, 1995).

The complex recycling pathways of MPRs require several sorting signals which have been mainly ascribed to structural determinants within the cytoplasmic domain. Efficient internalization of both MPRs depends on a tyrosine-containing motif (Canfield et al., 1991; Johnson et al., 1990). For MPR 300, this tyrosine was essential for binding of plasma membrane coated vesicle adaptors in vitro (Glickman et al., 1989). For MPR 46 a second, independently acting internalization signal contains two phenylalanine residues near to the membrane anchor (Johnson et al., 1990). Mannose 6-phosphate-dependent intracellular sorting of lysosomal enzymes requires a dileucine-containing motif near to the carboxy-terminal end of both MPRs, suggesting that the dileucine motif is part of a sorting signal for segregation of MPRs into clathrin-coated vesicles of the TGN (Johnson and Kornfeld, 1992a,b). Others have shown that phosphorylation of serine residues 82 and 157, the latter being located in close vicinity to the dileucine motif, is required for high-affinity binding of Golgi coated vesicle adaptors to the cytoplasmic domain of MPR 300 in vitro (LeBorgne et al., 1993). Little is known about signals involved in recycling from endosomes to the TGN and to the plasma membrane. Only recently, the tyrosine-containing peptide of MPR 46, which contributes to one of the internalization signals of the bovine receptor, was reported to be a critical determinant for return from the endosomal pathway to the TGN (Schulze-Garg et al., 1993). In situ-blockade of this epitope by peptide-specific antibodies resulted in accumulation of the receptor in a novel compartment which seems to be an intermediate for return from endosomes to the TGN.

Segregation of receptors into transport vesicles is mediated by binding of membrane-associated proteins, e.g. clathrin-coated vesicle adaptors, to sorting signals in their cytoplasmic domains (for review see Keen, 1990; Pearse and Robinson, 1990). Peptide-specific antibodies which bind to such signals ought to compete with adaptor binding and, as a consequence, interfere with sorting. Microinjection of peptide-specific antibodies into cultured cells can therefore be used to screen for sorting signals as shown by Schulze-Garg et al. (1993). Here we used microinjection of peptide-specific antibodies to investigate whether information for sorting is contained within the

carboxy-terminal peptides of the two MPRs, which show partial sequence homology including a cluster of acidic amino acids, a caseine kinase II phosphorylation site and a dileucine motif. Blockade of the carboxy-terminal peptide (residues 150-164) in the cytoplasmic domain of MPR 300 by peptide-specific antibodies resulted in redistribution from its main intracellular pool to a predominant plasma membrane localization. While, at steady state, MPR 300 was no longer detectable intracellularly in injected cells, internalization of anti-ectodomain antibodies was significant. The pattern of internalized anti-ectodomain antibodies suggested that residues 150-164 of MPR 300 are critical for efficient downstream transport within the early endosomal pathway. Blockade of the carboxy-terminal peptide of MPR 46 (residues 54-67) similarly exhausted the intracellular pool of this receptor, and transport of internalized anti-ectodomain antibodies was also slowed down within the early endosomal pathway. These data suggest that information for sorting during early steps in the endosomal pathway is contained within the carboxy-terminal peptides of the two MPRs.

MATERIALS AND METHODS

Antibodies and cell lines

Antibodies

MPR 300 was detected with affinity-purified rabbit antibody raised against MPR 300 from human placenta as described (von Figura et al., 1984), the monoclonal antibody 2C2 (Braulke et al., 1987), or an IgG fraction obtained from goat serum (Causin et al., 1988) by chromatography on Protein G-Sepharose (Pharmacia, Freiburg, Germany) equilibrated with 20 mM Na-phosphate, pH 6.5, according to the manufacturors advice. MPR 46 was detected with affinity-purified polyclonal antibody against MPR 46 from human placenta raised in rabbit (Stein et al., 1987) or goat (Wenk et al., 1991) or the monoclonal antibody 10C6 (K. Lohrengel and A. Hille-Rehfeld, unpublished observations). For each of the MPRs the immunofluorescence patterns obtained with antibodies from different species were indistinguishable. As indicated in the figure legends, antibodies for immunofluorescence labelling were chosen to exclude interference with other antibodies used in double or triple labelling experiments. TGN was labelled with monoclonal antibody 100/3 against γ-adaptin (Ahle et al., 1988). Lysosomes were labelled with polyclonal antiserum against cathepsin D raised in goat (Hasilik and Neufeld, 1980). Internalized transferrin was detected with rabbit anti-human transferrin (Janssen Biochemica, Beerse, Belgium).

Antiserum against residues 150-164 of human MPR 300 was as described (Delbrück et al., 1994). Rabbit antiserum against residues 49-67 of human MPR 46 was obtained by immunization of the synthetic peptide conjugated to keyhole limpet hemocyanin as described (Messner et al., 1989; Nadimpalli et al., 1991). Peptide-specific antibodies were purified from both sera by affinity chromatography on immobilized peptides as described (Geuze et al., 1984; Nadimpalli et al., 1991).

Cell lines

Human skin fibroblasts were obtained for diagnostic purpose and cultured as described (Sly and Grubb, 1979). Human hepatoma cells, HepG2, were cultured as described (Stoorvogel et al., 1989). Cells were periodically tested for the absence of mycoplasma by Hoechst stain (Serva, Heidelberg, Germany).

Preparation of F_{ab} fragments

Affinity-purified immunoglobulins from rabbit or goat (1 mg) were digested for 1 or 36 hours, respectively, with papain (20 μg) at 37°C as described (Hille et al., 1992). Rabbit F_{ab} fragments were purified by chromatography on Protein A-Sepharose (Pharmacia, Freiburg, Germany), and goat F_{ab} fragments were purified by molecular sieving chromatography on Superdex G75 (Pharmacia, Freiburg, Germany) using phosphate buffered saline (PBS) as eluent.

Metabolic labelling and immunoprecipitation

Cultures that were 80% confluent on 5.5 cm Petri dishes were labelled for 15 hours with 11 MBq of [^{35}S]methionine and harvested as described (Punnonen et al., 1996). An extract of cell membranes in phosphate buffered saline containing 0.5% Triton X-100 was used for immunoprecipitation of MPR 46 or MPR 300 as described (Punnonen et al., 1996; Lemansky et al., 1985). Where indicated, peptide-specific antibodies were preincubated with an excess of free peptide for 1 hour on ice. For competition experiments with anti-MPR 300CT, the cell extract was preadsorbed on immobilized MPR 300CT-peptide prior to immunoprecipitation, to remove unspecific aggregates of cellular proteins which were induced by peptide MPR 300CT. Immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970) and fluorography (Bonner and Laskey, 1974).

Microinjection and internalization of endosomal markers

Cells were grown on polylysine-coated coverslips for 2 days before microinjection of F_{ab} fragments at 1-5 mg/ml (Hille et al., 1992). Within this concentration range, apparently identical results were obtained. After 0.5-30 hours incubation at 37°C, cells were either fixed for indirect immunofluorescence or loaded with endosomal markers as described (Schulze-Garg et al., 1993) with the modifications specified below.

Monoclonal antibody 10C6 against MPR 46 (0.2 mg/ml) or goat F_{ab} fragment against MPR 300 (0.5 mg/ml) were offered for internalization at 37°C for up to 2 hours in Eagle's minimum essential medium containing 10 mM Hepes-KOH, pH 7.4, and 5 mg/ml bovine serum albumine (BSA).

Human holo-transferrin (Sigma, Deisenhofen, Germany) was offered at 10 μg/ml in Eagle's minimum essential medium containing 10 mM Hepes-KOH, pH 7.4, and 5 mg/ml BSA for 5-30 minutes at 37°C.

Indirect immunofluorescence

Immunofluorescence staining of cells was performed as described (Punnonen et al., 1996). Injected F_{ab} fragments were detected with AMCA-conjugated donkey anti-rabbit Ig. Compartment-specific markers were routinely detected with FITC-conjugated donkey anti-mouse Ig and Texas Red-conjugated donkey anti-goat Ig. For triple-staining of injected rabbit F_{ab} fragment, endocytosed goat anti-MPR 300 F_{ab} and transferrin, rabbit anti-transferrin Ig (whole molecule) and FITC-conjugated donkey anti-goat Ig were first bound simultanously. Free arms of the anti-goat Ig were then blocked by a 30 minute incubation with preimmune goat Ig (100 μg/ml in PBS containing 0.5% saponin), before anti-transferrin Ig and injected F_{ab} fragments were labelled with Texas Red-conjugated goat anti-rabbit F_c and AMCA-conjugated donkey anti-rabbit Ig.

For staining of plasma membrane MPRs, cells were fixed and incubated with rabbit F_c-fragments (200 μg/ml in PBS containing 0.2% gelatin) for 30 minutes to block F_c-receptors. After binding of anti-MPR antibodies (200 μg/ml in PBS containing 0.2% gelatine) for 1 hour, cells were permeabilized and processed for immunofluorescence as above.

Confocal laser scanning microscopy was performed as described (Hille et al., 1992; Schulze-Garg et al., 1993). Figures present overlays of 800 nm optical sections comprising the whole depth of the cells.

Immunogold labelling of cryosections

Immuno-electron microscopy was performed as described (Harding

and Geuze, 1992; Slot et al., 1991). HepG2 cells were fixed for 1 hour with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Cells were then scraped and embedded in 10% gelatine. After infiltration with 1.7 M sucrose and 15% polyvinylpyrrolidone (M_r 10,000) in 0.1 M sodium phosphate buffer for 24 hours, ultrathin cryosections were cut and picked up with a drop of 1% methyl cellulose in 1.2 M sucrose (Liou and Slot, 1994). Marker antibodies were diluted in PBS containing 0.1% BSA. Protein A conjugated with 5, 10 or 15 nm colloidal gold (kindly provided by Dr H. J. Geuze, Utrecht, The Netherlands) was diluted to an absorbence (520 nm) of 0.1, 0.2 or 0.3, respectively, with PBS containing 1% BSA (Slot and Geuze, 1985; Slot et al., 1991). Double- or triple labelling was performed by sequential incubations of the sections with marker antibodies and Protein A-gold. To stabilize the antibody-Protein A-gold complexes and to prevent interference with the following antibodies, sections were treated with 4% paraformaldehyde in PBS before incubation with the next antibody. Finally, sections were stained with 2% uranylacetate for 5-10 minutes, and then rinsed with 0.4% uranylacetate, 1.8% methylcellulose (Liou and Slot, 1994).

RESULTS

Characterization of peptide-specific antibodies

Synthetic peptides which correspond to the carboxy-terminal residues 150-164 of the cytoplasmic domain of MPR 300 or residues 54-67 of the cytoplasmic domain of MPR 46 (see Fig. 1) were used to affinity-purify peptide-specific antibodies from polyclonal rabbit sera. These antibody fractions were referred to as anti-MPR 300CT or anti-MPR 46CT, according to their antigenic reactivity with the carboxy-terminal residues of the two MPRs. As shown in Fig. 2A by immunoprecipitation from metabolically labelled cultured human fibroblasts or hepatoma cells (HepG2) anti-MPR 46CT and anti-MPR 300CT antibodies specifically recognized MPR 46 (1st lane) or MPR 300 (3rd to 6th lane), respectively. The authenticity of the two polypeptides recognized by the peptide-specific antibodies was confirmed by comparison with immunoprecipitates obtained with standard antibodies raised against whole MPR 46 (2nd lane) or MPR 300 (7th and 8th lane). The peptide-specific reactivity was corroborated by competition experiments. Preincubation of the tail-specific antibodies with the antigenic peptide efficiently abolished immunoprecipitation of metabolically labelled MPRs, whereas irrelevant peptides, which correspond to peptide sequences of the cytoplasmic domain close to the membrane anchor (residues 8-22 of MPR 46 and 18-37 of MPR 300), had no effect (Fig. 2B).

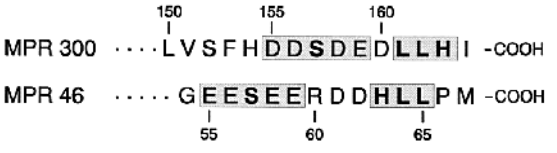

Fig. 1. Partial sequence homology of the carboxy-terminal peptides of MPR 300 (residues 150-164 of the cytoplasmic domain, starting with lysine 2,328 of MPR 300 as residue 1; Oshima et al., 1988) and MPR 46 (residues 54-67 of the cytoplasmic domain, starting with glutamine 211 as residue 1; Pohlmann et al., 1987). Shaded boxes indicate the caseine kinase II phosphorylation site and the dileucine motif.

Anti-MPR 300CT antibodies detect the receptor preferentially in Golgi membranes

The immunofluorescence pattern of MPR 300 in fixed and permeabilized cells was compared for staining with anti-MPR 300CT antibodies and an antibody raised against whole MPR 300 from human placenta, which does not react with the

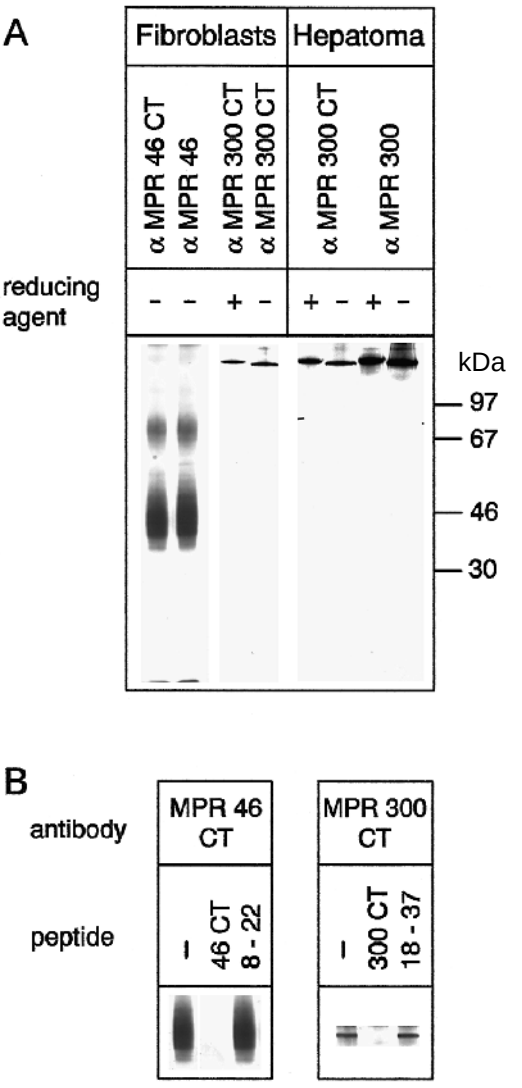

Fig. 2. Specificity of the peptide-specific antibodies. Human skin fibroblasts or hepatoma cells (HepG2) were metabolically labelled with [^{35}S]methionine. (A) An extract of cell membranes was used for immunoprecipitation with the peptide-specific antibodies against MPR 46CT (αMPR 46CT) or against MPR 300CT (αMPR 300CT). Control antibodies were against whole MPR 46 (αMPR 46) or whole MPR 300 (αMPR 300), both from rabbit. The immunoprecipitates were analyzed on non-reducing (-) or reducing (+) gels followed by fluorography. (B) MPR 46 or MPR 300 were immunoprecipitated from human skin fibroblasts with the peptide-specific antibodies against MPR 46CT or against MPR 300CT and analyzed as above. Where indicated, peptides MPR 46CT (46CT) or MPR 300CT (300CT), or peptides corresponding to amino acids 8-22 of the cytoplasmic tail of MPR 46 or to amino acids 18-37 of the cytoplasmic domain of MPR 300 were added at 350-fold molar excess over the peptide-specific antibody. The sections of the fluorogram containing immunopreciptated MPR 46 (left) or MPR 300 (right) are shown.

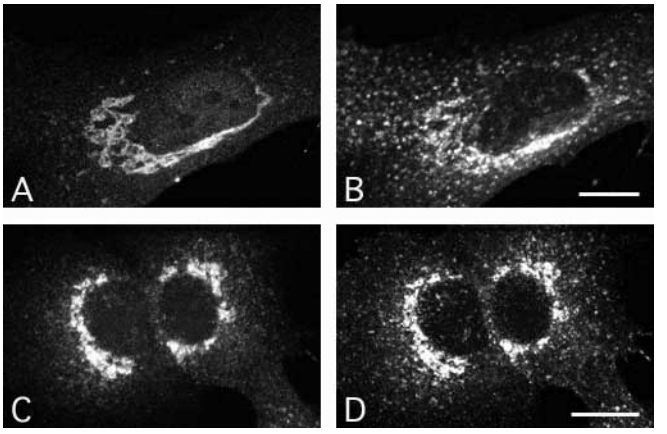

Fig. 3. The peptide-specific antibody against MPR 300CT selectively labels perinuclear structures. Human skin fibroblasts (A,B) or HepG2 cells (C,D) were fixed, permeabilized and processed for double immunofluorescence staining with antibodies against peptide MPR 300CT (A,C) and goat IgG against whole MPR 300 (B,D). Bars, 10 μm.

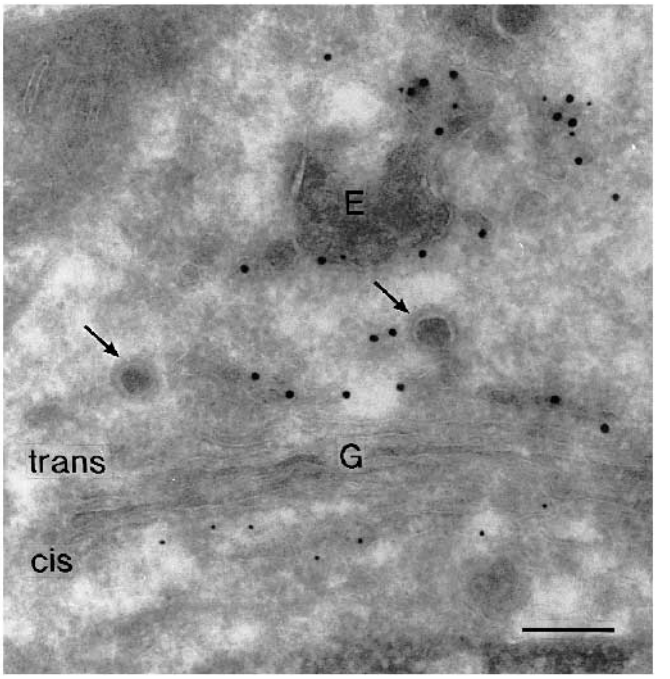

Fig. 4. Preferential labelling of *cis*-Golgi cisternae by the peptide-specific antibody against MPR 300CT. Ultrathin cryosections of human hepatoma cells (HepG2) were double labelled with antibodies against peptide MPR 300CT (10 nm gold) and whole MPR 300 raised in rabbit (15 nm gold). G, Golgi stack; E, endosome. Bar, 200 nm.

carboxy-terminal peptide of the receptor (A. Hille-Rehfeld, unpublished observation). The antibody against whole MPR 300 stained perinuclear, Golgi-like structures as well as peripheral endosomal vesicles of human fibroblasts (Fig. 3B) or HepG2 cells (Fig. 3D). In contrast, anti-MPR 300CT antibodies selectively stained perinuclear tubules in both cell types (Fig. 3A,C). To characterize the anti-MPR 300CT-positive structures at the ultrastructural level, immunogold labelling was performed on cryosections of HepG2 cells (Fig. 4). In the *cis*-Golgi cisternae immunogold labelling was found exclusively with anti-MPR 300CT antibodies (10 nm gold) but not with antibodies against whole MPR 300 (15 nm gold). The lack of *cis*-Golgi staining with the latter antibody can be explained either by its low affinity towards incompletely assembled MPR 300 (Sahagian, 1984) or by limited accessibility of the receptor in the lumen of *cis*-Golgi membranes. *Trans*-Golgi membranes and endosomal vacuoles were also labelled with anti-MPR 300CT antibodies but here, labelling was predominant for antibodies against whole MPR 300. In single labelling experiments with the two antibodies the same difference in the distribution of gold particles was obtained (not shown), therefore interference of the two antibodies can be excluded. The antibody against whole MPR 300 had been used earlier to quantitate MPR 300 in compartments of HepG2 cells (Klumperman et al., 1993), showing that less than 4% of the total labelling for MPR 300 resided within the Golgi stack. Our observation that immunogold labelling with anti-MPR 300CT antibody was at least as intensive at the *cis*-side of the Golgi than at the *trans*-side or in endosomes, suggests that the anti-MPR 300CT epitope was partially blocked in the TGN and endosomes.

Antibody-induced redistribution of MPR 300

F_{ab} fragments against MPR 300CT were injected into cultured human fibroblasts, and cells were incubated at 37°C for 0.5 to 30 hours, to allow binding of injected antibodies to the cytoplasmic domain of MPR 300. Cells were then fixed and permeabilized to investigate the steady state distribution of MPR 300 by immunofluorescence staining with an antibody against

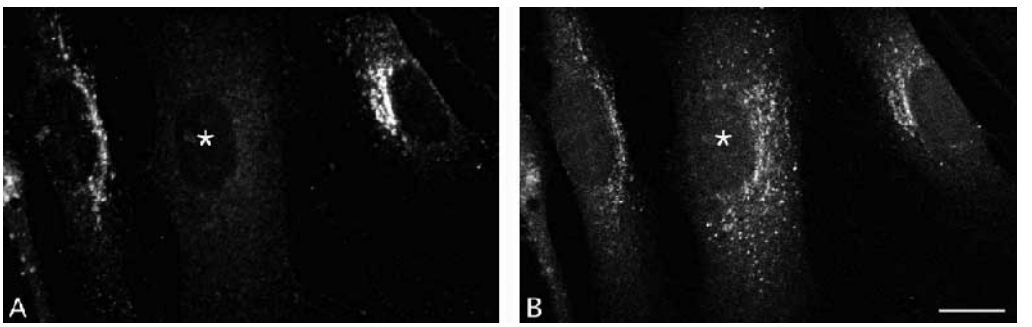

Fig. 5. Antibody-induced redistribution of MPR 300. Human skin fibroblasts were injected with F_{ab} fragments against MPR300CT (5 mg/ml). At 90 minutes after injection, cells were fixed, permeabilized and processed for immunofluorescence staining with goat anti-MPR 300 (A) and mouse anti-MPR 46 (B). The injected cell (asterisk) had been identified by immunofluorescence staining of injected F_{ab} fragments. Bar, 20 μm.

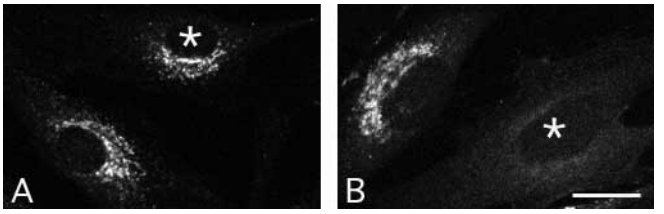

Fig. 6. Coinjection of antigenic peptide prevents antibody-induced redistribution of MPR 300. Human skin fibroblasts were injected with F_{ab} fragments against MPR 300CT (1.7 mg/ml) together with 18 mg/ml of peptide MPR 300CT (A), or a peptide corresponding to residues 18-37 of the cytoplasmic domain of MPR 300 (B). At 90 minutes after injection, cells were fixed, permeabilized and processed for immunofluorescence staining with goat anti-MPR 300. Injected cells (asterisks) were identified by immunofluorescence staining of injected F_{ab} fragments. Bar, 20 μm.

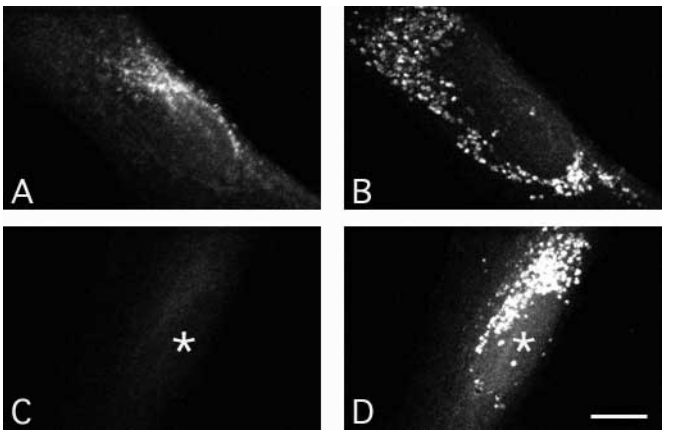

Fig. 7. Redistributed MPR 300 is not missorted to lysosomes. Human skin fibroblasts were treated with leupeptin and pepstatin (final concentration 100 μM each) for 18 hours, and then injected with F_{ab} fragments against MPR 300CT (5 mg/ml). After a 90 minute incubation in the continuous presence of protease inhibitors, cells were fixed, permeabilized and processed for immunofluorescence staining with mouse anti-MPR 300 (A,C) and cathepsin D (B,D). (A,B) Non-injected control cell; (C,D) injected cell (asterisks) that was identified by immunofluorescence staining of injected F_{ab} fragments. Bar, 10 μm.

its luminal domain, which does not interfere with binding of injected antibodies to the cytoplasmic domain. Injected cells were identified by immunodetection of injected rabbit F_{ab} fragment. In non-injected control cells, MPR 300 was found predominantly in perinuclear tubules and vesicles (Fig. 5A), which also contain MPR 46 (Fig. 5B) and have been shown earlier to localize in close vicinity to TGN-derived coated vesicles (Schulze-Garg et al., 1993). Additional peripheral vesicles partly colocalized with endosomal markers (see below, Fig. 9A,B). As soon as 30 minutes after injection of F_{ab} fragments against MPR 300CT, MPR 300 was no longer detectable in intracellular structures, the effect being stable for at least 30 hours (shown for 90 minutes post injection, Fig. 5A, cell labelled with asterisk). Antibody-induced redistribution of MPR 300 was completely abolished by coinjection of a 350-fold molar excess of antigenic peptide over the injected F_{ab} fragment (Fig. 6A). In contrast, an irrelevant peptide did not inhibit at the same concentration (Fig. 6B). This result corroborates the peptide-specific reactivity of the F_{ab} fragment. Distribution of MPR 46 (Fig. 5B) or γ-adaptin from Golgi coated vesicles (not shown) was not affected in injected cells, indicating that the overall structure of the TGN and endosomes was not disturbed.

To investigate whether antibody-tagged MPR 300 was missorted to and rapidly degraded within lysosomes, cells were treated with the protease inhibitors leupeptin and pepstatin, which have been shown earlier to prevent degradation of mutant MPR 46 when missorted to lysosomes (Peters et al., 1990). The presence of protease inhibitors in the culture medium during 18 hours before microinjection and throughout until fixation of cells did not prevent the loss of MPR 300 from intracellular structures of injected cells (Fig. 7C). Lysosomes, which were heavily labelled for cathepsin D (Fig. 7B,D), did not contain detectable levels of MPR 300 either in controls (Fig. 7A,B) or in injected cells (Fig. 7C,D).

To investigate whether MPR 300 was redistributed from its main intracellular localization to the plasma membrane, immunofluorescence staining of non-permeabilized cells was performed. When compared to non-injected control cells, the signal of MPR 300 at the surface of injected cells was significantly increased as shown by binding of antibodies against the luminal domain either to fixed but non-permeabilized cells (Fig. 8A), or to non-fixed cells that had been cooled on ice to abolish internalization (not shown). Cell surface MPR 300 showed a dot-like pattern in injected cells as in control cells, suggesting clustering of the receptor in coated pits (shown for an injected cell with high magnification in Fig. 8B).

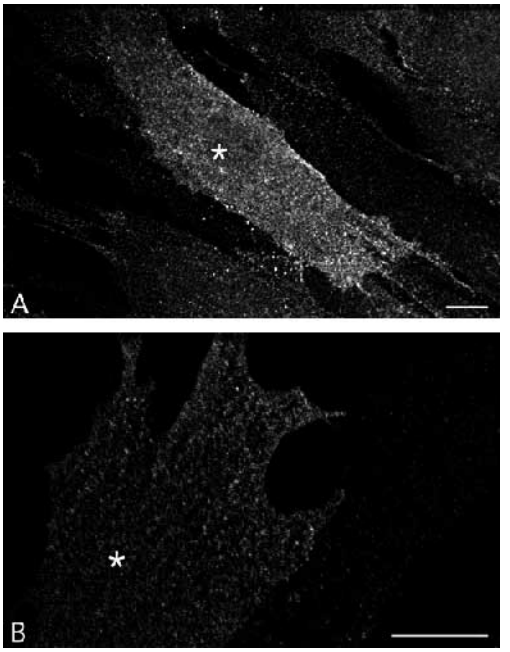

Fig. 8. Antibody-induced accumulation of MPR 300 at the cell surface. Human skin fibroblasts were injected with F_{ab} fragments against MPR 300CT (5 mg/ml). At 90 minutes after injection, cells were fixed and processed for immunofluorescence staining of cell surface MPR 300 with goat IgG. The injected cells (asterisks) were identified by immunofluorescence staining of injected F_{ab} fragments. Bars, 10 μm.

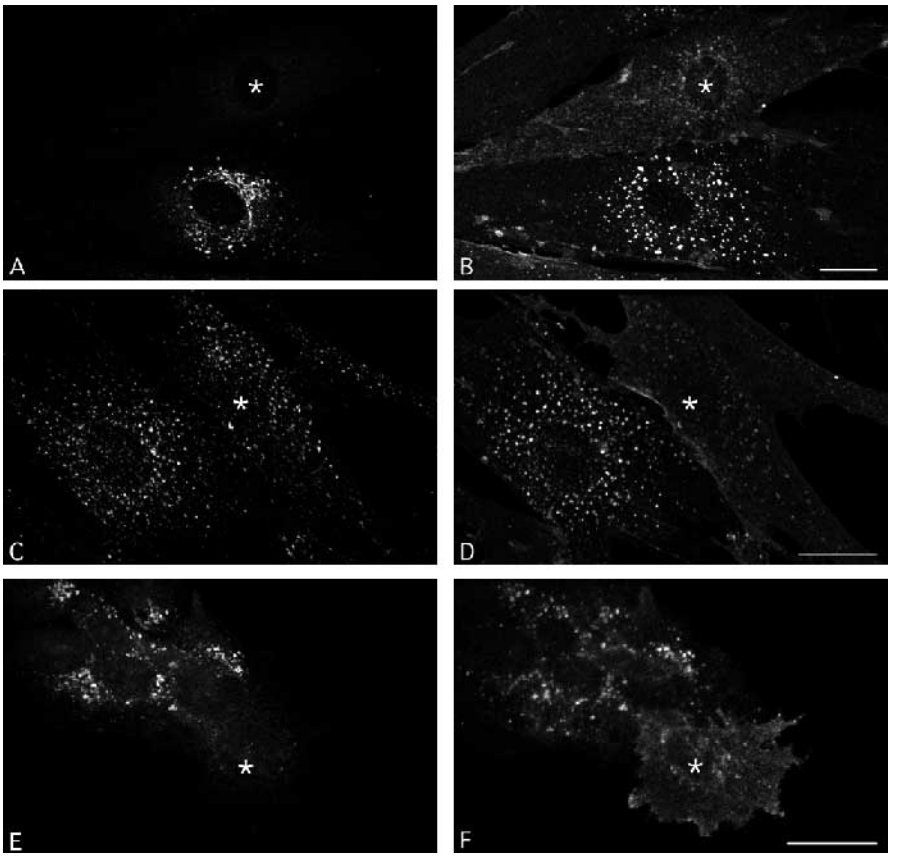

Fig. 9. Downstream transport of MPR 300 in the endocytic pathway is slowed down by anti-MPR 300CT antibodies. Human skin fibroblasts (A-D) or HepG2 cells (E,F) were injected with F_{ab} fragments against MPR 300CT (5 mg/ml). At 90 minutes after injection, cells were allowed to internalize F_{ab} fragments against the luminal domain of MPR 300 (0.5 mg/ml, raised in goat) for 1 hour (A-D) or 2 hours (E,F) at 37°C. Cells shown in C and D were loaded with human transferrin (final concentration 10 μg/ml) during the last 15 minutes of F_{ab} internalization. Cells were then fixed, permeabilized and processed for immunofluorescence staining of total cellular MPR 300 with mouse IgG (A,E) or internalized transferrin (C), and internalized F_{ab} fragments against MPR 300 (B,D,F). Horizontally arranged pictures show the same sections. Injected cells (asterisks) were identified by immunofluorescence staining of injected F_{ab} fragments. Bars, 20 μm.

To investigate whether redistributed MPR 300 was blocked at the cell surface or whether it had retained its ability for internalization and rapid recycling, cells were incubated with F_{ab} fragments against the luminal domain of MPR 300 (referred to as anti-ectodomain antibodies) at 37°C for up to 120 minutes. During the first 20 minutes of a continuous internalization, anti-ectodomain antibodies were found in small vesicles which were randomly scattered throughout the cytoplasm, and which did not show apparent differences among control cells and injected cells (not shown). Upon prolonged incubation, internalized anti-ectodomain antibodies were then concentrated in larger perinuclear structures of control cells (shown for 60 of internalization, Fig. 9B,D). In contrast, staining of anti-ectodomain antibodies in injected cells was usually restricted to peripheral dots (Fig. 9D, cell labelled with asterisk), and perinuclear structures were observed only scarcely (Fig. 9B, cell labelled with asterisk). This pattern did not change for at least 2 hours of continuous internalization (not shown). The dot-like staining of internalized anti-ectodomain antibodies was detectable only when cells had been permeabilized prior to binding of secondary antibodies, suggesting that it reflects intracellular endocytic vesicles. Accumulation of anti-ectodomain antibodies apparently did not shift the steady state distribution of MPR 300 in injected cells, as the signal for MPR 300 within internal membranes still was below the limit of detection (Fig. 9A). This finding supports the view that redistributed MPR 300 was not immobilized at the plasma membrane, but continued to be internalized and recycled. Surprisingly, the intensity of internalized anti-ectodomain antibodies was much lower in injected cells compared to non-injected controls, despite the increased amount of anti-ectodomain antibodies bound to MPR 300 at the plasma membrane of injected cells. Taken together, these data suggest that MPR 300 was internalized in injected cells, but that some step in downstream transport along the endosomal pathway had become rate-limiting. The lower amount of internalized anti-ectodomain antibodies may reflect either rapid recycling or impaired internalization of MPR 300.

To rule out a general blockade in the endocytic pathway of injected cells, internalization of transferrin was investigated. Neither the pattern of internalized transferrin nor the intensity of fluorescence in transferrin-positive structures was significantly different in injected cells and non-injected controls, suggesting that endocytic traffic in general had not been affected (Fig. 9C). These results indicate that injected F_{ab} fragments against MPR 300CT selectively disturbed transport of MPR 300 within the endosomal pathway.

When F_{ab} fragments against MPR 300CT were injected into human hepatoma cells (HepG2), similar results were obtained: Steady state levels of intracellular MPR 300 were reduced below the limit of detection (Fig. 9E), and intracellular accumulation of internalized F_{ab} fragments against the lumenal domain of MPR 300 was slowed down (Fig. 9F). These results suggest that antibody-induced redistribution and disturbed endocytic transport of MPR 300 are not a peculiarity of human fibroblasts.

Antibody-induced redistribution of MPR 46

F_{ab} fragments against residues 54-67 of the cytoplasmic domain of human MPR 46 (F_{ab} fragments against MPR 46CT)

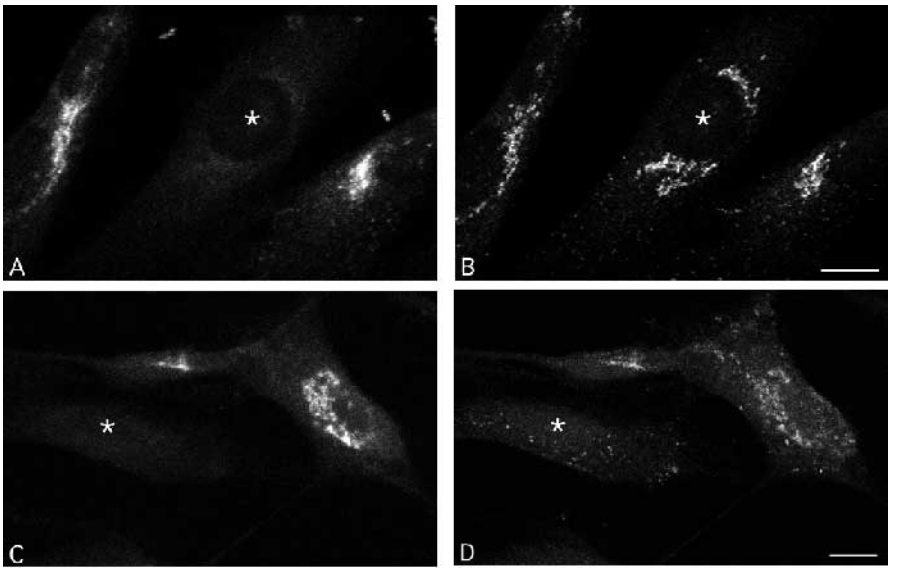

Fig. 10. Antibody-induced redistribution of MPR 46. Human skin fibroblasts were injected with F_{ab} fragments against MPR 46CT (2 mg/ml). At 90 minutes after injection, cells were either immediately fixed, permeabilized and processed for double immunofluorescence staining of total cellular MPR 46 with mouse IgG (A) and MPR 300 with goat IgG (B), or loaded with a mouse monoclonal antibody against the luminal domain of MPR 46 (0.2 mg/ml) for 2 hours at 37°C before fixation, permeabilization and immunofluorescence staining of total cellular MPR 46 with goat Ig (C) and the internalized anti-MPR 46 antibodies (D). Injected cells (asterisks) were identified by immunofluorescence staining of injected F_{ab} fragments. Bar, 10 μm.

were injected into human fibroblasts, and the intracellular localization of the receptor was investigated by immunofluorescence staining. F_{ab} fragments against MPR 46CT caused the receptor to vanish from its main perinuclear localization (Fig. 10A), similar to the results described above for antibody-induced redistribution of MPR 300. The effect was selective for MPR 46, with neither MPR 300 (Fig. 10B) nor γ-adaptin from coated vesicles of the TGN (not shown) being affected. Antibody-induced redistribution was complete by 30 minutes after microinjection, and the effect was stable for at least 30 hours. Missorting of redistributed MPR 46 to lysosomes was not detectable, even if protease inhibitors were added to prevent lysosomal degradation (not shown). At steady state, redistributed MPR 46 was not detectable at the cell surface of injected cells (not shown). As an indirect attempt to investigate the presence of MPR 46 at the cell surface and its ability to be internalized, a monoclonal antibody against the ectodomain of MPR 46 was continuously offered to the cells at 37°C for up to 2 hours. Internalized anti-ectodomain antibodies accumulated in small peripheral vesicles of injected cells as shown for the 2 hour internalization (Fig. 10D, cell labelled with asterisk). Under identical conditions, anti-ectodomain antibodies internalized by control cells reached perinuclear structures (Fig. 10D), which constitute the main intracellular localization of MPR 46 at steady state (Fig. 10C). The most likely explanation for these results is that anti-MPR 46CT antibodies caused redistribution of MPR 46 to the plasma membrane, from where it can be internalized and transported within the endosomal pathway at reduced rate. The failure to detect steady state levels of MPR 46 at the cell surface can be explained by the fairly low sensitivity of immunofluorescence detection of MPR 46, which does not allow detection of the receptor after dilution within the large area of the plasma membrane.

DISCUSSION

Antibody-induced redistribution of mannose 6-phosphate receptors

Both MPRs colocalize in all compartments of their recycling pathway, but differences in their steady state concentrations and lateral segregation within membranes have been observed (Klumperman et al., 1993, and references cited therein). Their main steady state localization is intracellular, less then 10% residing at the plasma membrane. In human fibroblasts, which have been used in the present study, MPR 46 is predominantly found in the TGN, whereas MPR 300 is found more frequently in perinuclear endosomes (Schulze-Garg et al., 1993).

The intracellular distribution of the two MPRs most likely reflects their overlapping but partially different functions in sorting (for review see Hille-Rehfeld, 1995). Since recycling of both MPRs essentially follows the same routes, they might carry related sorting signals for individual transport steps. As the C-terminal peptide of the cytoplasmic domain of the two MPRs shows partial sequence homologies including a serine residue within a caseine kinase II-like phosphorylation site, and a dileucine motif, we have now investigated the role of this peptide for sorting and recycling. Microinjection of F_{ab} fragments against the carboxy-terminal residues 150-164 of the cytoplasmic domain of MPR 300, or residues 54-67 of the cytoplasmic domain of MPR 46 resulted in complete depletion of the intracellular pools of either receptor as detected by immunofluorescence staining. The use of monovalent F_{ab} fragments precluded possible artefacts due to antibody-induced cross-linking. Moreover, redistributed receptors apparently were not missorted to and degraded within lysosomes, since after treatment of cells with protease inhibitors they did not colocalize with cathepsin D. For MPR 300 it was shown that the steady state distribution was shifted in favour of the plasma membrane, whereas MPR 46 was not detectable at the plasma membrane, either in controls or in microinjected cells. The latter is most likely due to the low sensitivity of cell surface immunofluorescence staining, since redistributed MPR 46, like MPR 300, was able to bind and internalize anti-ectodomain antibodies. Intracellular accumulation of internalized anti-ectodomain antibodies did not lead to detectable increase in the intracellular pool of redistributed mannose 6-phosphate receptor in injected cells, suggesting that recycling of redistributed receptor to the plasma membrane had not become a rate-limiting step.

Injection of F_{ab} fragments against MPR 46CT or against MPR 300CT selectively affected recycling of the receptor from which the antigenic peptide was derived, suggesting that despite the partial sequence homology of the antigenic peptides cross-reactivity with the second MPR was negligible. Neither the pattern of markers for TGN or endosomes nor receptor-mediated internalization of transferrin were affected in microinjected cells, suggesting that the overall structure of these compartments and general vesicular transport between them was not disturbed.

Peptide-specificity of the injected F_{ab} fragments was corroborated by immunoprecipitation in the presence or absence of competing peptides and, for MPR 300, also by coinjection of the antigenic peptide. The latter control was not possible to perform for peptide 54-67 of MPR 46, because its solubility in injection buffer was too low to be used in sufficient excess. For MPR 46 we have shown earlier that binding of control antibodies (directed against peptides 8-22 or 39-43 of the cytoplasmic domain) did not affect the intracellular distribution of the receptor, whereas antibodies against peptide 44-48 caused MPR 46 to accumulate within a novel endosomal subcompartment (Schulze-Garg et al., 1993). The distinct effects observed for the various antibodies used in the present and earlier study suggest that peptide-specific antibodies, despite their large size compared to the cytoplasmic domain of the receptors, can selectively mask epitopes which are relevant for intracellular sorting. Several attempts to obtain antibodies against additional epitopes in the cytoplasmic domain of MPR 300 failed. When a polyclonal antiserum raised against the whole cytoplasmic domain of MPR 300 (Rosorius et al., 1993) was used to subfractionate antibodies on immobilized peptides, the main antigenic epitope turned out to be contained within peptide 150-164. On the other hand, immunization of rabbits with several shorter peptides gave rise to peptide-specific antibodies which did not bind the native receptor. Nevertheless, since the effect of both anti-MPR CT antibodies on recycling of MPRs was very much alike, the conclusion appears to be legitimate that also the effect of anti-MPR 300CT antibodies reflects an epitope-specific blockade.

The carboxy-terminal peptides of mannose 6-phosphate receptors contain information for sorting in the early endosomal pathway

Antibody-induced redistribution of MPRs from their main intracellular localization to the plasma membrane suggests that binding of F_{ab} fragments to the carboxy-terminal peptide of their cytoplasmic domains interferes with rapid internalization or intracellular retention of the receptors. Internalization of redistributed MPRs was not abolished, as both receptors continued to bind and import anti-ectodomain antibodies. Despite the marked accumulation of MPR 300 at the plasma membrane, the amount of internalized anti-ectodomain antibodies in microinjected cells apparently was not increased over that observed in non-injected control cells. This result suggests that either the internalization rate was reduced in injected cells, or that internalized antibody-tagged receptors rapidly recycled to the plasma membrane, because an early step of downstream transport along the endosomal pathway had become rate-limiting. In the latter case, the rate of recycling to the plasma membrane must be assumed to exceed the rate of internalization, intracellular retention of MPRs being controlled by the efficiency of downstream sorting along the endosomal pathway. In contrast to non-injected cells, internalized anti-ectodomain antibodies did not reach late endosomal compartments nor the TGN of injected cells, suggesting that binding of F_{ab}-fragments against the carboxy-terminal peptide interferes with downstream transport along the endosomal pathway.

The semiquantitative nature of the immunofluorescence analysis does not allow us to decide whether the effect of F_{ab} fragments against the carboxy-terminal peptides is restricted to downstream intraendosomal transport, or whether internalization of receptors was also affected. From the published studies on mutational analysis of internalization signals, there is no evidence for involvement of the carboxy-terminal peptide sequence in endocytic traffic (Canfield et al., 1991; Johnson et al., 1990). On the other hand, the cytoplasmic domain of MPR 46 contains two independently acting internalization motifs (Johnson et al., 1990), suggesting that binding of coated vesicle adaptors or other steps required for clustering of MPRs into coated pits may be regulated by a complex interaction of multiple epitopes. Therefore, one could imagine that the carboxy-terminal peptide of MPRs exerts an auxilliary function in regulation of rapid internalization.

Antibody-induced redistribution of MPRs raised the question of whether the carboxy-terminal peptide of MPRs constitutes a sorting signal in the sense that it binds cytoplasmic proteins such as clathrin-coated vesicle adaptors. Alternatively, this peptide motif might influence sorting of receptors by stabilizing the three-dimensional structure of its cytoplasmic domain in a way that signals elsewhere in the protein are exposed or modulated for binding of cytoplasmic proteins. Finally, it cannot be excluded that antibody-induced blockade might be the result of steric hindrance, if the carboxy-terminal peptide was brought in close vicinity to a sorting signal elsewhere in the cytoplasmic domain by three-dimensional folding. On the other hand, a first hint that cytoplasmic proteins which mediate sorting may in fact bind to the carboxy-terminal peptide of MPRs comes from the observation that the MPR 300CT epitope displays differential accessibility to antibodies among various intracellular compartments. In this context it seems intriguing that the anti-MPR 300CT epitope is best accessible at the *cis*-side of the Golgi, but mostly blocked in the *trans*-Golgi network and endosomes, i.e. in those compartments where binding of cytoplasmic proteins may be involved in the control of sorting and recycling. However, we cannot exclude the possibility that blockade of the anti-MPR 300CT epitope in TGN and endosomes reflects a conformational effect. The immunofluorescence pattern obtained with anti-MPR 46CT antibodies did not provide evidence for compartment-selective binding. Therefore, if the carboxy-terminal peptides constitute a signal which is recognized by cytoplasmic proteins, than their affinity towards MPR 46 must be lower than towards MPR 300.

While this work was in progress, other investigators reported that the carboxy-terminal peptide of the two MPRs contains information for sorting into clathrin-coated pits of the *trans*-Golgi network: a Leu-Leu-His motif in the cytoplasmic domain of MPR 300 (residues 161-164) and a His-Leu-Leu motif in the cytoplasmic domain of MPR 46 (residues 63-65) was required for correct intracellular sorting of newly synthesized lysosomal enzymes, providing indirect evidence that these sequences might constitute the recognition signal for binding

of adaptor proteins from Golgi coated vesicles (AP1-adaptors) (Johnson and Kornfeld, 1992a,b). In addition, Hoflack and coworkers have shown that a peptide corresponding to the cytoplasmic domain of MPR 300 efficiently competes for binding of AP1-adaptors to permeabilized cells only when serine residues 82 and 157 were phosphorylated, the latter being part of the peptide MPR 300CT (LeBorgne et al., 1993). These results raised the question of whether binding of F_{ab} fragments against MPR CT might block exit of MPRs from the TGN by interfering with segregation into clathrin-coated vesicles. We never observed blockade of MPRs in the TGN with any of our antibody preparations. We therefore assume that, if an epitope for binding of AP1-adaptors has been masked, MPRs which are tagged with F_{ab} fragments leave the TGN together with the bulk flow of the secretory pathway (Pfeffer and Rothman, 1987).

Conclusion

The results of the present and earlier studies show that the carboxy-terminal peptide sequences of MPRs play a critical role in several sorting steps during recycling. While sorting in the TGN is thought to be regulated by a dileucine motif in the carboxy-terminal 15mer peptide, it remains to be established which amino acids are involved in endocytic trafficking. Future work will be aimed at studying the interaction of coated vesicle adaptors or endosome-associated proteins with peptides corresponding to the carboxy-terminal sequences of MPRs.

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REFERENCES

Ahle, S., Mann, A., Eichelsbacher, U. and Ungewickell, E. (1988). Structural relationships between clathrin assembly protein from the Golgi and the plasma membrane. *EMBO J.* **7**, 919-929.

Bonner, W. M. and Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83-87.

Braulke, T., Gartung, C., Hasilik, A. and von Figura, K. (1987). Is movement of mannose 6-phosphate specific receptor triggered by binding of lysosomal enzymes? *J. Cell Biol.* **104**, 1735-1742.

Canfield, W. M., Johnson, K. F., Ye, R. D., Gregory, W. and Kornfeld, S. (1991). Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insulin-like growth factor II receptor to amino acids 24-29 of the cytoplasmic tail. *J. Biol. Chem.* **266**, 5682-5688.

Causin, C., Waheed, A., Braulke, T., Junghans, U., Maly, P., Humbel, R. E. and von Figura, K. (1988). Mannose 6-phosphate/insulin-like growth factor II-binding proteins in human serum and urine. *Biochem. J.* **252**, 795-799.

Delbrück, R., Desel, D., von Figura, K. and Hille-Rehfeld, A. (1994). Proteolytic processing of cathepsin D in prelysosomal organelles. *Eur. J. Cell Biol.* **64**, 7-14.

Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Peppard, J., von Figura, K., Hasilik, A. and Schwartz, A. L. (1984). Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell* **37**, 195-204.

Glickman, J. N., Conibear, E. and Pearse, B. M. (1989). Specificity of binding of clathrin adaptors to signals on the mannose 6-phosphate/insulin-like growth factor II receptor. *EMBO J.* **8**, 1041-1047.

Harding, C. V. and Geuze, H. J. (1992). Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listera monocytogenes for presentation to T cells. *J. Cell Biol.* **119**, 531-542.

Hasilik, A. and Neufeld, E. (1980). Biosynthesis of lysosomal enzymes in fibroblasts. *J. Biol. Chem.* **255**, 4937-4945.

Hille, A., Klumperman, J., Geuze, H. J., Peters, C., Brodsky, F. M. and von Figura, K. (1992). Lysosomal acid phosphatase is internalized via clathrin-coated pits. *Eur. J. Cell Biol.* **59**, 106-115.

Hille-Rehfeld, A. (1995). Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochem. Biophys. Acta* **1241**, 177-194.

Johnson, K. F., Chan, W. and Kornfeld, S. (1990). The cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. *Proc. Nat. Acad. Sci. USA* **87**, 10010-10014.

Johnson, K. F. and Kornfeld, S. (1992a). A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. *J. Biol. Chem.* **267**, 17110-17115.

Johnson, K. F. and Kornfeld, S. (1992b). The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.* **119**, 249-257.

Keen, J. H. (1990). Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* **59**, 415-438.

Klumperman, J., Hille, A., Veenendaal, T., Oorschot, V., Stoorvogel, W., von Figura, K. and Geuze, H. J. (1993). Differences in the endosomal distribution of the two mannose 6-phosphate receptors. *J. Cell Biol.* **121**, 997-1010.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Le Borgne, R., Schmidt, A., Mauxion, F., Griffiths, G. and Hoflack, B. (1993). Binding of AP-1 Golgi adaptors to membranes requires phosphorylated cytoplasmic domains of the mannose 6-phosphate/insulin-like growth factor II receptor. *J. Biol. Chem.* **268**, 22552-22556.

Lemansky, P., Gieselmann, V., Hasilik, A. and von Figura, K. (1985). Synthesis and transport of lysosomal acid phosphatase in normal and I-cell fibroblasts. *J. Cell Biol.* **101**, 824-829.

Liou, W. and Slot, J. W. (1994). Improved fine structure in immunolabeled cryosections after modifying the sectioning and pick-up conditions. *Int. Congress Elect. Microsc.* **13**, Paris, 17-22 July.

Messner, D. J., Griffiths, G. and Kornfeld, S. (1989). Isolation and characterization of membranes from bovine liver which are highly enriched in mannose 6-phosphate receptors. *J. Cell Biol.* **108**, 2149-2162.

Nadimpalli, S. K., Schmidt, B., von Figura, K. and Hille, A. (1991). Antibodies against the cytoplasmic tail can differentiate between the quarternary forms of M_r 46,000 mannose 6-phosphate receptor. *FEBS Lett.* **280**, 61-64.

Oshima, A., Nolan, C. M., Kyle, J. W., Grubb, H. J. and Sly, W. S. (1988). The human cation-independent mannose 6-phosphate receptor. Cloning and sequence of the full-length cDNA and expression of functional receptor in COS cells. *J. Biol. Chem.* **263**, 2553-2562.

Pearse, B. M. F. and Robinson, M. S. (1990). Clathrin, adaptors and sorting. *Annu. Rev. Cell Biol.* **6**, 151-171.

Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and von Figura, K. (1990). Targeting of a lysosomal membrane protein: A tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* **9**, 3497-3506.

Pfeffer, S. R. and Rothman, J. E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* **56**, 829-852.

Pohlmann, R., Nagel, G., Schmidt, B., Stein, M., Lorkowski, G., Krentler, C., Cully, J., Meyer, H. E., Grzeschik, K. H., Mersmann, G., Hasilik, A. and von Figura, K. (1987). Cloning of a cDNA encoding the human cation-dependent mannose 6-phosphate-specific receptor. *Proc. Nat. Acad. Sci. USA* **84**, 5575-5579.

Punnonen, E.-L., Wilke, T., von Figura, K. and Hille-Rehfeld, A. (1996). The oligomeric state of 46 kDa mannose 6-phosphate receptor does not change upon intracellular recycling and binding of ligands. *Eur. J. Biochem.* **237**, 809-818.

Rosorius, O., Issinger, O. G. and Braulke, T. (1993). Phosphorylation of the cytoplasmic tail of the 300 kDa mannose 6-phosphate receptor is required for the interaction with a cytosolic protein. *J. Biol. Chem.* **268**, 21470-21473.

Sahagian, G. G. (1984). The mannose 6-phosphate receptor: function, biosynthesis and translocation. *Biol. Cell* **51**, 207-214.

Schulze-Garg, C., Böker, C., Nadimpalli, S., von Figura, K. and Hille-Rehfeld, A. (1993). Tail-specific antibodies that block return of 46,000 M_r mannose 6-phopshate receptor to the trans-Golgi network. *J. Cell Biol.* **122**, 541-551.

Slot, J. W. and Geuze, H. J. (1985). A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38**, 87-93.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991). Immunolocalization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* **113**, 123-135.

Sly, W. S. and Grubb, J. (1979). Isolation of fibroblasts from patients. *Meth. Enzymol.* **58**, 444-450.

Stein, M., Braulke, T., Krentler, C., Hasilik, A. and von Figura, K. (1987). 46 kDa mannose 6-phoshpate-specific receptor: biosynthesis, processing, subcellular location and topology. *Biol. Chem. Hoppe-Seyler* **368**, 937-947.

Stoorvogel, W., Geuze, H. J., Griffith, J. M., Schwartz, A. L. and Strous, G. J. (1989). Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein, and mannose 6-phosphate in human hepatoma cells. *J. Cell Biol.* **108**, 2137-2148.

von Figura, K., Gieselmann, V and Hasilik, A. (1984). Antibody to mannose 6-phosphate receptor induces receptor deficiency in human fibroblasts. *EMBO J.* **3**, 1281-1286.

Wenk, J., Hille A. and von Figura, K. (1991). Quantitation of M_r 46,000 and M_r 300,000 mannose 6-phosphate receptors in human cells and tissues. *Biochem. Int.* **23**, 723-732.

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