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Targeted disruption of the M_r 46 000 mannose 6-phosphate receptor gene in mice results in misrouting of lysosomal proteins

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Lysosomal enzymes containing mannose 6-phosphate recognition markers are sorted to lysosomes by mannose 6-phosphate receptors (MPRs). The physiological importance of this targeting mechanism is illustrated by I-cell disease, a fatal lysosomal storage disorder caused by the absence of mannose 6-phosphate residues in lysosomal enzymes. Most mammalian cells express two MPRs. Although the binding specificities, subcellular distribution and expression pattern of the two receptors can be differentiated, their coexpression is not understood. The larger of the two receptors with an M_r of ~300 000 (MPR300), which also binds IGFII, appears to have a dominant role in lysosomal enzyme targeting, while the function of the smaller receptor with an M_r of 46 000 (MPR46) is less clear. To investigate the *in vivo* function of the MPR46, we generated MPR46-deficient mice using gene targeting in embryonic stem cells. Reduced intracellular retention of newly synthesized lysosomal proteins in cells from MPR46 −/− mice demonstrated an essential sorting function of MPR46. The phenotype of MPR46 −/− mice was normal, indicating mechanisms that compensate the MPR46 deficiency *in vivo*.

Key words: gene targeting/MPR46 −/− mice/M_r 46 000 mannose 6-phosphate receptor/sorting of lysosomal enzymes

Introduction

Mannose 6-phosphate receptors (MPRs) mediate the targeting of newly synthesized soluble lysosomal enzymes. The receptors recognize mannose 6-phosphate residues which are specifically added to lysosomal enzymes on the synthetic route through the Golgi apparatus. Receptor–ligand complexes are formed in the trans-Golgi network and are sorted to a prelysosomal compartment, where they dissociate due to acidic pH. The ligands are delivered to lysosomes, while the MPR can recycle to the Golgi apparatus (for review see Kornfeld and Mellman, 1989; Kornfeld, 1992).

Two MPRs are known to mediate the transport of newly synthesized mannose 6-phosphate-containing ligands from the Golgi apparatus to prelysosomal structures of the endocytic pathway. The larger of the two receptors with an apparent molecular mass of 300 000 (MPR300) also mediates the endocytosis of mannose 6-phosphate-containing ligands from the plasma membrane and is a multifunctional receptor in mammalian cells that additionally binds the insulin-like growth factor II (IGFII) (Morgan *et al.*, 1987). The physiological function of the IGFII binding is unknown. The smaller MPR with an apparent molecular mass of 46 000 (MPR46) fails to endocytose ligands but mediates the secretion of a fraction of its ligands (Chao *et al.*, 1990).

All mammalian cell lines and tissues analysed contain both MPRs except for a few tumour-derived cell lines that lack MPR300 (Gabel *et al.*, 1983; Mainferme *et al.*, 1985). In the MPR300-deficient cells, 60–70% of the total newly synthesized lysosomal enzymes are secreted. Transfection of these cells with MPR46 decreased the secreted fraction to only ~50% (Watanabe *et al.*, 1990; Ma *et al.*, 1991). Overexpression of MPR46 in cells with normal levels of endogenous MPR300, e.g. BHK21, mouse L (Chao *et al.*, 1990) and MDCK cells (R.Bresciani, personal communication) increased the secreted fraction of newly synthesized lysosomal enzymes from 10 to ~50%. Thus, the view has emerged that MPR46 delivers its ligands to a site (early endosomes or plasma membrane) from where the ligands are released from the cells as well as transported to lysosomes (Chao *et al.*, 1990; Kornfeld, 1992). Inhibition of MPR46 by receptor-specific antibodies in cells with endogenous MPR300 did not increase the secretion of newly synthesized lysosomal enzymes (Stein *et al.*, 1987; Chao *et al.*, 1990). These results suggested that MPR46 plays only a minor role in targeting of newly synthesized lysosomal enzymes to lysosomes or that loss of its function can be compensated for by other receptors such as the MPR300.

To investigate the *in vivo* function of the MPR46 we generated MPR46-deficient mice. The present study clearly demonstrates that the complete loss of MPR46 is associated with a misrouting of mannose 6-phosphate-containing polypeptides out of the cell. This strongly argues for an essential function of MPR46 in the targeting of newly synthesized lysosomal enzymes and that this function cannot be compensated for by the endogenous MPR300.

Results

Generation of MPR46 −/− mice

The targeting vector described in Figure 1 was used to create a null-allele at the MPR46 locus in embryonic stem cells by positive/negative selection with G418 and gancyclovir according to Mansour *et al.* (1988). Out of 50 selected ES cell clones analysed by Southern blotting, nine clones exhibited homologous recombination at the MPR46 locus. The recombination, initially detected after *Eco*RI digestion of ES cell DNA and hybridization with the 5′ external probe A, was confirmed by digestion with *Bgl*II and probing with the 3′ external probe B (Figure 1). Four of these targeted ES cell clones (53, 120, 131 and 133) were used for microinjection into C57BL/6J blastocysts. A total of 10 chimeric mice, one female and nine males, were generated. The chimeric males were mated to non-Agouti C57BL/6J females. Four of the nine male chimeras generated from ES

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cell clones 120 and 133 had Agouti offspring and were shown by Southern blot analysis of tail DNA of their Agouti offspring to transmit the targeted MPR46 allele through the germ line. Mice heterozygous for the MPR46 gene disruption appeared normal. A total of 154 F_2 offspring generated by heterozygote crosses were genotyped by Southern blot analysis (see Figure 2A). A distribution of 34% +/+, 47% +/− and 19% −/− mice was detected

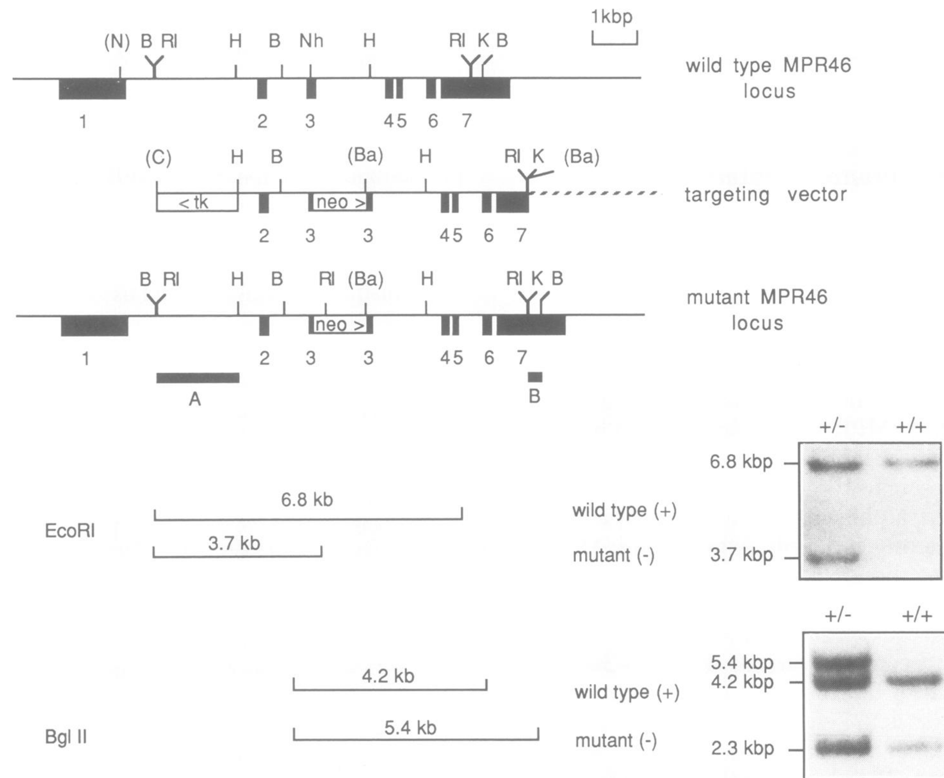

Fig. 1. Strategy for disruption of the MPR46 gene in mouse embryonic stem (ES) cells. **Upper panel.** Maps of the wild-type murine MPR46 locus (Ludwig *et al.*, 1992), the targeting vector and of the mutant MPR46 locus are given. The filled boxes represent numbered exons, solid lines intron and flanking sequences, the broken line plasmid vector pBluescript SK−; neo, neomycin resistance gene cassette (Thomas and Capecchi, 1987); tk, HSV-thymidine kinase gene cassette (Mansour *et al.*, 1988). Arrowheads mark direction of transcription for neomycin resistance and thymidine kinase gene. Restriction endonuclease sites used for cloning and Southern blot analysis of ES cell clones are shown (N, *Not*I; B, *Bgl*II; RI, *Eco*RI; H, *Hind*III; Nh, *Nhe*I; K, *Kpn*I; C, *Cla*I; Ba, *Bam*HI). A, 5′ external probe; B, 3′ external probe. **Bottom panel.** Expected DNA fragments of wild-type and mutant alleles after digestion of ES cell DNA with *Eco*RI and *Bgl*II were hybridized with probes A and B, respectively. Inserts: Southern blot analysis of genomic DNA from wild-type E-14 ES cells (+/+) (Hooper *et al.*, 1987) and an ES cell clone with a targeted allele (+/−) digested with *Eco*RI (upper insert) or *Bgl*II (lower insert). The sizes of hybridizing DNA fragments are given on the left. The 2.3 kbp DNA fragment is a non-specific DNA fragment recognized by probe B.

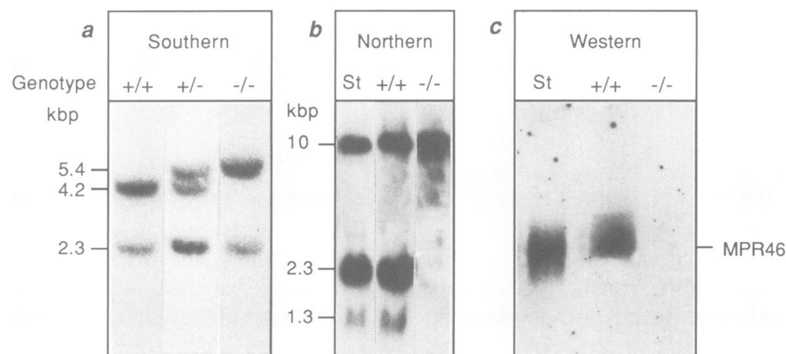

Fig. 2. Southern, Northern and Western blot analysis of MPR46 gene-deficient mice. (**a**) Southern blot analysis of genomic DNA, 10 μg, from MPR46 −/−, +/− and +/+ mice digested with *Bgl*II was performed as described in Materials and methods. The 4.2 kbp wild-type is replaced in the MPR46 −/− mouse by the recombinant 5.4 kbp fragment. All mice analysed are descendants of MPR46 recombinant ES cell clone 120. (**b**) MPR46 mRNA expression in MPR46 −/− and +/+ mice. 15 μg of total RNA prepared from mouse tissues (brain, lung, kidney, intestine, liver and testes) were analysed by Northern blotting using a 1:1 mixture of ^{32}P-labelled riboprobes specific for MPR46 and MPR300 as described (Matzner *et al.*, 1992; shown for kidney). RNA of LTK$^-$ mouse fibroblasts was used as a standard (St). The hybridizing 10 kbp RNA species corresponds to the MPR300 and the 2.3 and 1.3 kbp RNAs to MPR46 mRNA (Matzner *et al.*, 1992). MPR46 mRNA was absent in all MPR46 −/− tissues. (**c**) MPR46 protein expression in a Western blot. Membrane fractions of kidney and lung from MPR46 −/− and +/+ mice were analysed with a MPR46-specific polyclonal antiserum (shown for kidney). 100 ng of MPR46 purified from mouse liver was used as standard (St). In tissues of MPR46 −/− mice, no MPR46 protein was detectable.

(see Table I). MPR46 −/− mice contained neither detectable amounts of MPR46 mRNA nor MPR46 protein (Figure 2B and C).

MPR46 −/− mice do not display a discernible phenotype

MPR46 −/− mice were fertile and lacked phenotypic changes visible upon inspection or autopsy at the age of 3−4 months. Signs of lysosomal storage were not detectable by light and electron microscopic examination of a variety of tissues including liver, kidney and brain (not shown). In serum and tissues (liver, kidney, brain, heart and testes), the activities of up to five lysosomal enzymes known to be transported via mannose 6-phosphate receptors (β-hexosaminidase, β-galactosidase, α- and β-mannosidase, β-glucuronidase) were within the range of controls (Table II).

Table I. Genotype of offspring from heterozygote crosses

ES line	+/+	+/−	−/−	Total
120	7	13	8	28
133	45	60	21	126
	52 (34%)	73 (47%)	29 (19%)	154

The genotype of offspring was determined by Southern blot analysis of tail DNA as described in Materials and methods.

Table II. Lysosomal enzyme activities in serum of MPR46 +/+, +/− and −/− mice

	Genotype		
	+/+ (n = 17)	+/− (n = 17)	−/− (n = 16)
β-Hexosaminidase[a]	8.23 ± 3.98	9.44 ± 3.82	9.19 ± 3.25
β-Galactosidase[b]	41.0 ± 26	55.0 ± 29	43.0 ±17
α-Mannosidase[b]	500 ± 244	484 ± 293	482 ± 171
β-Mannosidase[b]	172 ± 65	148 ± 29	155 ± 44.8

Activities are expressed in units × 10^{-3} ([a]) or units × 10^{-6} ([b]) per ml serum, where one unit of enzyme cleaves 1 μmol of substrate/min. Activities were determined as described in Materials and methods.

Lysosomal enzymes are misrouted in cells of MPR46 −/− mice

Earlier studies indicated an MPR46-mediated secretion of lysosomal enzymes from the cells. Since the serum levels of lysosomal enzymes appeared to be in the range of controls in MPR46 −/− mice (Table II), the targeting of these enzymes was studied in primary cell cultures from MPR46-deficient mice. Freshly isolated thymocytes and splenocytes or fibroblasts grown from the retroperitoneal connective tissue were metabolically labelled and their secretions analysed for the presence of mannose 6-phosphate-containing polypeptides by MPR300 affinity chromatography. This method detects the mannose 6-phosphate-containing lysosomal enzyme precursors as a group (Chao *et al.*, 1990). A 3- to 5-fold increase of mannose 6-phosphate-containing polypeptides in secretions of MPR46 −/− cells was observed (Figure 3). It is a common observation that cultured cells, including fibroblasts, secrete ~10% of their newly synthesized lysosomal enzyme precursors (Kornfeld, 1992). Thus, up to 50% of the newly synthesized lysosomal enzyme precursors may become secreted in MPR46 −/− cells. Weak bases such as ammonium chloride induce the secretion of newly synthesized lysosomal enzyme precursors by inhibiting their binding to MPRs in the trans-Golgi network (von Figura and Hasilik, 1986). The pattern of mannose 6-phosphate-containing polypeptides in secretions from MPR46 −/− splenocytes and NH_4Cl-treated MPR46

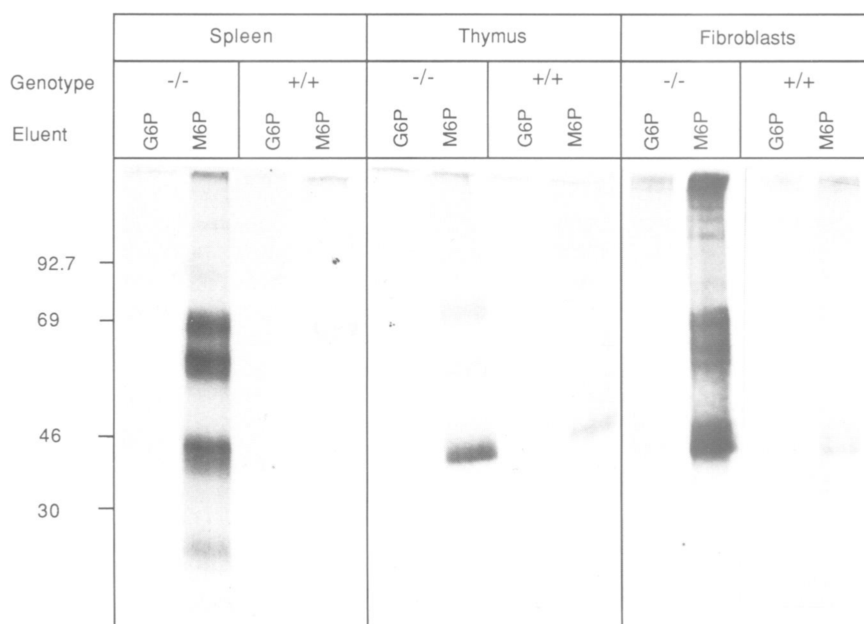

Fig. 3. Secretion of mannose 6-phosphate-containing proteins from spleen and thymus cells and cultured fibroblasts from MPR46 −/− and +/+ mice. Freshly isolated spleen and thymus cells of male mice and cultured fibroblasts were metabolically labelled with [^{35}S]methionine. The media were passed over a MPR300 Affigel 10 column. Non-specifically bound material was eluted with 5 mM glucose 6-phosphate (G6P). The material specifically bound to the column was eluted with 5 mM mannose 6-phosphate (M6P) and separated by SDS−PAGE. Molecular weight standards are given on the left. Based on densitometry the secretions of MPR46 −/− cells contained a 3- to 5-fold higher amount of M6P-containing proteins than those of MPR46 +/+ cells.

−/− and +/+ splenocytes was comparable (not shown), which may indicate that deficiency of MPR46 does not cause secretion of a subgroup of lysosomal enzymes.

The increased secretion of lysosomal enzymes could be demonstrated directly for β-hexosaminidase. Fibroblasts were incubated for 36 h in medium supplemented with 5 mM mannose 6-phosphate to prevent re-uptake of the secreted β-hexosaminidase by the MPR300. The β-hexosaminidase in the secretions, expressed as a percentage of the total activity in secretions plus cells, was 19% in MPR46 +/+ and 55% in MPR46 −/− fibroblasts.

Discussion

The physiological role of the MPR46 for the transport of lysosomal enzymes has remained an enigma mainly because mammalian cells express simultaneously two MPRs. Only a few tumour-derived cell lines that have lost the expression of the MPR300 are an exception to this rule (Gabel *et al.*, 1983; Mainferme *et al.*, 1985). In these cells the greater part of newly synthesized lysosomal enzymes is secreted. Secretion is even further enhanced in these cells when the endogenous MPR46 is functionally inactivated by blocking antibodies, and secretion can be reduced, in part, by overexpression of MPR46 (Stein *et al.*, 1987; Watanabe *et al.*, 1990; Ma *et al.*, 1991). From these observations it was concluded that MPR46 participates in the transport of newly synthesized lysosomal enzymes to lysosomes. However, neither the endogenous MPR46 nor its overexpression can compensate for the loss of MPR300. On the contrary, functional inactivation of MPR46 in cells that express both MPRs such as fibroblasts or hepatocytes has no effect on secretion, suggesting that the endogenous MPR300 can compensate for a loss of MPR46 (Stein *et al.*, 1987). Based on these observations the prediction was that loss of MPR46 is not likely to impair the sorting of newly synthesized lysosomal enzymes.

The lack of a discernible phenotype of MPR46 −/− mice generated in this study and the normal levels of lysosomal enzymes in organs and serum of these mice were in agreement with the predictions deduced from the earlier cell culture studies. Surprisingly, primary cells and fibroblasts derived from MPR46 −/− mice showed an increased secretion of mannose 6-phosphate-containing polypeptides incuding the lysosomal enzyme β-hexosaminidase. This strongly argues for an essential function of MPR46 in targeting of newly synthesized lysosomal enzymes from the secretory pathway to the endocytic route. Preliminary results indicate that *in vivo* the increased secretion of newly synthesized lysosomal enzymes is corrected by their re-uptake through endocytic receptors. When re-uptake by MPR300 and mannose receptors is blocked by a bolus of mannose 6-phosphate and mannan the level of several lysosomal enzymes, including arylsulfatase A and β-galactosidase, increases significantly in the serum of MPR46 −/− mice over that in MPR46 +/+ mice (A.Köster, unpublished results). Under cell culture conditions the grossly expanded extracellular space and the limited availability of endocytic receptor systems may compromise the re-uptake of secreted lysosomal enzymes and uncover their increased secretion. If lysosomal catabolism in MPR46 −/− mice depends on the reinternalized lysosomal enzymes, insufficient re-uptake in some tissues or cells would produce signs of lysosomal storage. The normal ultrastructural appearance of lysosomes in all tissues of MPR46 −/− mice examined, indicates that the intralysosomal enzyme levels are high enough to prevent lysosomal storage. Thus either the residual targeting in the absence of MPR46 and/or the re-uptake of secreted enzymes sustains in MPR46 −/− mice intralysosomal enzyme levels sufficient for normal catabolic function of lysosomes.

It is not entirely clear why earlier cell culture studies did not reveal the essential role of MPR46 for targeting of newly synthesized lysosomal enzymes. Blocking antibodies may have failed to reveal this essential function due to an incomplete inactivation of MPR46 by this procedure. The failure of endogenous MPR300 to compensate the loss of MPR46 in the knock-out mice may indicate that the amount of MPR300 is insufficient to compensate for a complete loss of MPR46. However, it is also conceivable that the two receptors act in sequence along the sorting route (the MPR46 having a major role in the trans-Golgi network and the MPR300 at the plasma membrane and within the endosomal route) or transport different subsets of lysosomal enzymes. If the amount of MPR300 is limiting, overexpression of MPR300 in MPR46 −/− should correct the missorting of lysosomal enzymes. The pattern of ligands in secretions from MPR46 −/− splenocytes and MPR46 +/+ splenocytes treated with NH_4Cl was similar. This indicates that the selective loss of MPR46 and the general inhibition of MPR-dependent sorting of lysosomal enzymes by NH_4Cl induces the secretion of identical lysosomal enzymes. However, it cannot be excluded that the two receptors transport subpopulations of one lysosomal enzyme which differ by their affinities for the two receptors.

The enhanced secretion of newly synthesized lysosomal enzymes in MPR300-deficient cell lines and the inability to correct the secretion by overexpression of MPR46 has shown the critical role of MPR300 for lysosomal enzyme targeting. Its additional role as a clearing receptor for IGFII in mammalian species appears to be responsible for the lethal effect that can be associated with its loss. The MPR300 gene is maternally imprinted in mice (Barlow *et al.*, 1991). Only the maternal copy is transcriptionally active, while the paternal copy becomes inactivated after zygote formation. Inheritance of deletions that include the MPR300 gene (e.g. T^{hp} or t^{Lub2}) from the mother can be associated with prenatal death of mice during embryogenesis. The lethal effect is probably due to a gene dosage effect of IGFII, since disruption of the IGFII gene, which is paternally imprinted, can restore vitality in MPR300-deficient mice (Filson *et al.*, 1993). However, there is no information about the levels of lysosomal enzymes and IGFII in tissues and serum of these mice.

Disruption of the MPR46 gene has clearly shown its critical role for targeting of lysosomal enzymes in cells and in mice. Preliminary studies indicate that in mice its loss can be compensated for by other endocytic receptors. It remains to be determined whether on a cellular level, loss of MPR46 can be compensated for by increased levels of MPR300, whether the receptors transport different groups of lysosomal enzymes, whether the receptors feed different subpopulations of endosomes/lysosomes and why the receptors are expressed in a tissue-specific manner. Resolution of these issues will require complementary studies in mice or cell lines deficient in either or both MPRs.

Materials and methods

Targeting vector

A genomic clone (λG13) containing the MPR46 gene was isolated from a genomic library of mouse PCC4 teratocarcinoma cells derived from 129/SvJ mice. A 7.8 kbp *Not*I–*Kpn*I DNA fragment of λG13 containing the MPR46 gene was subcloned into pBluescript SK–. A 1.2 kbp *Xba*I DNA fragment derived from pMC1Neo-polyA (Thomas and Capecchi, 1987) containing the neo^r cassette, was inserted into the unique *Nhe*I restriction site in exon 3. A 3.8 kbp *Hin*dIII–*Bam*HI fragment containing exon 2, the 5'part of exon 3 and the neo^r cassette and a 5.4 kbp *Bam*HI DNA fragment containing the 3' part of exon 3 and exons 4–7 were subsequently subcloned into a pBluescript SK– plasmid into which a 2 kbp *Hin*dIII–*Xho*I DNA fragment of pIC19R/MC1-TK (Mansour *et al.*, 1988) containing the tk cassette, had been inserted. The resulting targeting vector was linearized with *Cla*I, purified and used for electroporation of ES cells.

Embryonic stem cells

E14 ES cells (Hooper *et al.*, 1987) were cultured on neomycin resistant feeder layers in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 15% fetal calf serum (Boehringer, Mannheim), 1 × non-essential amino acids, 0.1 mM β-mercaptoethanol and 1 mM sodium pyruvate. Introduction of targeting vector DNA into ES cells was done by electroporation (Hooper *et al.*, 1987). Forty-eight hours after transfection, cells were selected in medium supplemented with 0.33 mg/ml G418 and 2 μM gancyclovir for ~12–14 days. Single colonies were analysed by Southern blotting as described below.

Blastocyst injection and generation of chimeric mice

Cells for microinjection were washed, trypsinized and resuspended in 10 mM HEPES-buffered DMEM containing 15% fetal calf serum. Blastocysts were collected on the day of injection by flushing uterine horns 3.5 days post-coitum with DMEM containing 15% fetal calf serum. 12–15 ES cells were injected into each blastocyst and were then implanted into the uterine horns of pseudopregnant $B6CBF_1$ mice, mated 2.5 days before with vasectomized MORO males (BRL, Füllinsdorf, Switzerland).

Southern and Northern hybridizations

High molecular genomic DNA was isolated from tails of 3 week old mice (Murphy and Hanson, 1987). Total RNA from mouse tissues was prepared from 6–8 week old mice as previously described (Matzner *et al.*, 1992). Hybond N (Amersham) was used as the hybridization membrane. The conditions for transfer, hybridization and washing were those recommended by the supplier. For Southern hybridization (Sambrook *et al.*, 1989), the ^{32}P-labelled probe was either a purified 0.24 kbp *Eco*RI–*Bgl*II fragment from the mouse MPR46 cDNA (3' external probe) or a 1.8 kbp *Hin*dIII–*Eco*RI fragment isolated from the mouse genomic clone (5' external probe). ^{32}P-labelled RNA probes for Northern blot analysis (Sambrook *et al.*, 1989) were obtained by *in vitro* transcription of receptor gene fragments cloned into the pGEM1 vector as described (Matzner *et al.*, 1992).

Western blot analysis

Membrane fractions derived from 200 μg total cellular protein were prepared and subjected to Western blot analysis as described (Wenk *et al.*, 1991). The blot was probed with a rabbit antiserum specific for the 66 amino acids of the cytoplasmic domain of MPR46 (dilution 1:100). Analysis was performed using an ECL light-based immunodetection system (Amersham).

Metabolic labelling of cells

Spleen and thymus were homogenized in medium A (50% MEM/50% RPMI 1640 containing 20 mM HEPES pH 7.2 and 5% heat inactivated, dialysed fetal calf serum), cells were pelleted for 2 min at 500 *g* and washed once in medium A. After incubation in medium B (MEM free of methionine containing 5% of fetal calf serum) for 45 min at 37°C, the cells were pelleted and resuspended in medium B (10^8 cells in 3 ml medium) containing 7.4 MBq [^{35}S]methionine for 5 h and chased for 3 h. Fibroblasts on confluent 3 cm dishes were incubated in 0.6 ml medium B for 1 h at 37°C and subsequently incubated in 2 ml medium B containing 4.4 MBq [^{35}S]methionine for 15 h. Media were processed as described (Chao *et al.*, 1990).

Lysosomal enzyme assays

For fluorometric assays 20 μl serum (diluted 1:10 or 1:100) were incubated with 0.2 ml 0.1 M sodium citrate, pH 4.6 containing the corresponding 4-methylumbelliferyl substrate (1 mM) at 37°C. After incubation (45 min up to 12 h) the reaction was stopped with 2 ml 0.2 M glycine, pH 10.4, and the 4-methylumbelliferyl fluorescence was measured.

Chromatography on MPR300 affinity column

Media obtained after labelling were applied to a 0.4 ml MPR300 affinity column as described (Chao *et al.*, 1990). The mannose 6-phosphate eluate was precipitated with 10% trichloroacetic acid overnight, dissolved in 60 μl 0.4 M Tris and heated for 5 min at 95°C in 10 mM dithiothreitol, 1% SDS and analysed by SDS–PAGE (10% polyacrylamide) and fluorography.

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Note added in proof

During the preparation of this manuscript similar results were reported independently by the group of B.Hoflack, EMBL, Heidelberg (submitted).