

A chimera of the cytoplasmic tail of the mannose 6-phosphate/IGF-II receptor and lysozyme localizes to the TGN rather than prelysosomes where the bulk of the endogenous receptor is found

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SUMMARY

We fused the cytoplasmic and transmembrane domains of the bovine mannose 6-phosphate/IGF-II receptor (MPR) to lysozyme, a monomeric secretory protein thought to be devoid of sorting information. When the resulting chimera (lys/MPR) was transiently expressed in COS cells or stably expressed in CV1 cells, it had a predominantly intracellular distribution in the *trans*-Golgi region, with less than 10% present on the surface. In contrast, a similar chimera containing the transmembrane and cytoplasmic domains of the low density lipoprotein receptor (lys/LDLR) was localized to the plasma membrane, even though it endocytoses efficiently. Exchanging domains between the lys/MPR and lys/LDLR chimeras indicated that the MPR cytoplasmic domain contains the information necessary to specify

the intracellular localization of the chimeric molecule. This signal must be located in the membrane-proximal third of the tail, as deletion of the last 120 residues of the 163 residue tail has no obvious effect on the distribution of lys/MPR.

However, the recycling of the lys/MPR does not completely mimic that of the intact endogenous MPR, as immunofluorescence labelling shows that they are predominantly in different locations, indicating a role for the luminal domain of the MPR in determining the steady-state distribution of the MPR itself.

Key words: *trans*-Golgi network, coated pit, sorting

INTRODUCTION

The cation-independent mannose 6-phosphate/IGF-II receptor (MPR) binds mannose 6-phosphate residues on lysosomal enzymes as these newly synthesized molecules arrive in the *trans*-Golgi network (TGN). Here the receptor is recruited into clathrin-coated pits containing the HA-1 adaptor, thus sequestering the lysosomal enzymes from bulk secretory components for efficient delivery to lysosomes. The MPR releases its ligand in an acidified endosome (prelysosome) and returns to the TGN for multiple rounds of delivery. Also, when MPR reaches the plasma membrane it is efficiently endocytosed in clathrin-coated pits containing the HA-2 adaptor (for review of coated pit components see Pearse and Robinson, 1990; Ahle et al., 1988) and recycles back through endosomes and the TGN (for reviews see Kornfeld, 1992; Kornfeld and Mellman, 1989; von Figura, 1991; Klumperman et al., 1993).

Thus, depending on the cell type, the MPR follows an unusual transport pathway that requires multiple transport signals. The MPR cytoplasmic tail has a well defined endocytosis signal Y²⁴KY²⁶SKV²⁹ (Lobel et al., 1989; Jadot et al., 1992). This region also apparently acts as a signal for the efficient sorting of lysosomal enzymes from the TGN separately or in combination with the C-terminal residues LLHV¹⁶³ (Johnson and Kornfeld, 1992). Both types of coated vesicle

adaptor have been shown to bind to the MPR tail. Recognition by the HA-2 plasma membrane adaptor is abolished by mutation of the tyrosines important for the endocytosis signal but these same mutations do not appear to abolish sorting (Johnson and Kornfeld, 1992) or the binding of the HA-1 Golgi adaptor (Glickman et al., 1989).

In order to see if the cytoplasmic tail could specify the intracellular distribution of a heterologous protein, we decided to construct chimeric molecules by fusing portions of the MPR to lysozyme, a protein not expected to have dominant sorting signals. Chicken lysozyme was chosen because it is monomeric, readily secreted when expressed in heterologous systems, able to tolerate C-terminal extensions and provides a convenient antigenic tag (Kondor-Koch et al., 1985; Munro and Pelham, 1987).

We expressed the hybrid proteins, and truncated or mutated derivatives, in cultured cells and looked at their steady-state distribution by immunofluorescence. In contrast to a similar hybrid protein containing lysozyme and the cytoplasmic portion of the low density lipoprotein receptor (lys/LDLR) found largely on the plasma membrane, the lysozyme/MPR hybrid (lys/MPR) was located predominantly in an intracellular compartment, most likely the TGN. However, the localization of lys/MPR did not closely mimic that of the intact, endogenous MPR found largely in prelysosomes.

MATERIALS AND METHODS

Construction of vectors for the expression of chimeras

COS cell expression vectors SAY1 (Munro and Pelham, 1987) and HYK (Pelham et al., 1988) containing the chicken lysozyme coding sequence and related vectors were kindly provided by S. Munro and H. Pelham in this laboratory. The expression vector CDM8 (Seed, 1987) was used to create stable cell lines in combination with RSV neo carrying the neomycin resistance gene (de Wet et al., 1987).

lys/LDLR and lys/MPR

The pL3 plasmid, containing cDNA encoding the human LDLR, was obtained from the American Type Culture Collection (ATCC no. 57004/57005). The complete LDLR coding sequence was cleaved from pL3 using a 5' *Hind*III site and a *Sma*I site located in the 3' untranslated sequence (bp 2818), and ligated into pHYK that had been cut with *Hind* III and *Sma*I. The resulting vector, pLDLR, directs the expression of the human LDLR in COS cells.

A plasmid containing sequence 3500-4647 of the bovine MPR inserted into the *Sac*I site of pUC19 (pE5; Lobel et al., 1989) was kindly provided by S. Kornfeld, Washington University, St Louis, MS).

Fusion of lysozyme and receptor sequences took advantage of the termination codon of the lysozyme cDNA in pSAY1 forming part of the *Xba*I site. By cutting with *Xba*I and blunting with mung bean nuclease, the stop codon as well as one other base at the 3' end of the coding sequence is removed. *Bam*HI sites at position 2337 in the LDLR sequence and at position 3811 in the MPR sequence were created by site-directed mutagenesis in M13mp18 by the method of Zoller and Smith (1984). When these *Bam*HI sites were cut and blunted with mung bean nuclease, an extra base was left at their 5' ends. This allowed fusion of the lysozyme sequence with each receptor sequence, restoring the last residue of the lysozyme sequence and maintaining the reading frame. Thus fragments carrying receptor sequences cut with *Bam*HI, treated with mung bean nuclease and cut with *Eco*RI were ligated into SAY1 that had been cut with *Xba*I, blunted, and cut with *Eco*RI. The resulting vectors, pI/M and pI/L, are able to direct the expression of lys/MPR and lys/LDLR chimeras in COS cells. Further mutations or stop codons were introduced by site directed mutagenesis by the method of Kunkel (1985) or by PCR (Clackson, 1991). Mutated fragments were sequenced, either in M13mp18 or pUC before inserting into SAY1.

pCDM8 expression vectors

Fragments encoding lys/MPR and mutants were released from pI/M with *Hind*III and *Not*I and ligated into pCDM8 that had been similarly cut. The lys/LDLR coding sequence was released from pI/L as a *Hind*III-*Sma*I fragment. It was ligated into pCDM8, which had been cut with *Pst*I, end-filled, and cut with *Hind*III (thus destroying the *Pst*I site). The expression vectors thus created are pCDM8 (I/M), pCDM8 (Δ 2Y), pCDM8 (1401), pCDM8 (1355), pCDM8 (1333) and pCDM8 (I/L).

Transfection of tissue culture cells

Cells were grown at 37°C under 10% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Transient transfection was achieved by a procedure involving calcium phosphate precipitation of the DNA. COS7 cells to be transfected were plated the night before to 50-70% confluence, and given fresh medium 1 hour before transfection. For each 75 cm² flask, 25 µg of PEG-purified DNA and 63 µl of 2.5 M CaCl₂ was added to 0.563 ml of TE/10 (1 mM Tris-HCl, pH 8.0, 0.01 mM EDTA). While bubbling air through the solution with a pasteur pipette, 0.625 ml of 2× HBS (25 mM HEPES, pH 7.12, 1.5 mM Na₂HPO₄, 280 mM NaCl) was added dropwise, then left for 20 minutes at room temperature to complete the precipitation. This was added to the tissue culture

medium, and cells were gassed and incubated overnight. The cells were then rinsed with DBPS, given fresh medium, and processed the following day e.g. for immunofluorescence studies.

Alternatively, cells were transiently transfected in the presence of chloroquine. Typically, cells were grown to 75% confluence in a 25 cm² flask. After washing the cells twice with serum-free DMEM, 0.5 ml of serum-free DMEM and 5 µl of plasmid DNA (approx 0.5 mg/ml) were added to the flask, followed by 0.5 ml of 1 mg/ml DEAE-dextran in TBS. After a 30 minute incubation, the medium was replaced with DMEM containing 10% FCS and 100 µg/ml chloroquine, and the cells were incubated for a further 3 hours. The cells were incubated for 2 days in DMEM + 10% FCS before harvesting. Such cells were used for endocytosis assays.

For the selection of stable transfectants, the calcium phosphate technique was used to co-precipitate 20 µg of pCDM8 expression vector with 1 µg of pRSVneo. The precipitate was added to a 75 cm² flask of CV-1 cells (one flask per plasmid) and left overnight. The cells were washed and given fresh medium the next day (day 1); on day 2 they were split 1:5 into 100 cm² plates, and on day 3 the cells were put under Geneticin G418 selection (Gibco; 400 µg/ml active concentration). The G418-containing medium was replaced every 3 days, and resistant colonies picked 20 days following transfection. Some transfected cell lines were subcloned by limiting dilution (Harlow and Lane, 1988). The chimeric proteins were found to have long half lives in CV1 cells. Metabolic labelling of cells was performed with a pulse of [³⁵S]methionine followed by continued incubation in the presence of unlabelled methionine. Calculating the rates of degradation from densitometry of immunoprecipitated bands at different time points gave estimated half lives of 10-20 hours for each of the chimeras lys/1333, lys/1355 and lys/MPR.

Antibodies

The following monoclonal antibodies were used: D1.3, anti-chicken egg white lysozyme (Amit et al., 1986) a mouse IgG1,κ, provided by Jeff Foote; F10 (F10-6-18), anti-chicken egg white lysozyme, provided by R. Poljiak and Sean Munro; 1B5, a mouse IgG, which recognizes a luminal epitope on an unidentified transmembrane protein that appears to be found in prelysosomes and lysosomes (provided by Mark Marsh, MRC Laboratory for Molecular Cell Biology, University College London); C7, anti-LDLR (Beiseigel et al., 1982), which recognizes a luminal epitope near the LDL binding site and was provided by E. Gheradi; and 9E10, mouse monoclonal, which recognizes a peptide epitope from human c-myc (Munro and Pelham, 1987) provided by M. Lewis.

Also the following polyclonal antisera were used: rabbit anti-lysozyme, raised against chicken egg white lysozyme was provided by M. Lewis; anti-JG2 (rat 4.1 and rabbit 130), raised against a fusion protein containing the bovine MPR cytoplasmic domain (Glickman et al., 1989). It recognizes the bovine MPR, but shows little if any cross-reactivity to the human or simian MPR and staining is only seen in cells expressing the bovine MPR tail sequence; rabbit anti-galactosyltransferase, raised against human β-1,4-galactosyltransferase, provided by Eric Berger (Roth and Berger, 1982); and rabbit anti-MPR (286), raised against the bovine MPR, purified from liver (Glickman et al., 1989). It recognizes human, simian, bovine and rodent MPR. Only the affinity-purified antiserum recognizes the MPR in simian cells fixed with methanol, although the staining pattern is the same with crude antiserum and formaldehyde fixations.

Sheep anti-mouse (as well as donkey anti-rabbit) antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red (TR) were obtained from Amersham. These antibodies are affinity-purified and absorbed against human, rat and rabbit (or mouse) IgG, so they show no cross-reactivity and can be used for double labelling studies.

FITC-conjugated goat anti-rat IgG (Sigma) was run through a rabbit IgG column to remove any cross-reacting antibody. It recognizes both mouse and rat IgG.

Immunofluorescence microscopy

Cells were split onto sterile 4-well coated microscope slides (Hendley-Essex) and allowed to spread overnight in a drop of medium before being rinsed quickly in DPBS and fixed in one of three ways:

(1) glutaraldehyde fixation. The slides were immersed in a solution of 2% paraformaldehyde, 0.1% glutaraldehyde in PBS at RT for 30 minutes, followed by permeabilization for 10 minutes with 0.5% Triton X-100. Free aldehydes were blocked by a 10 minute incubation in 1 mg/ml NaBH₄ in PBS. The slides were rinsed with PBS between each step, and stored at 4°C overnight in PBS if necessary.

(2) Paraformaldehyde fixation. Slides were immersed in a freshly prepared solution of 4% paraformaldehyde in PBS for 30 minutes at RT, rinsed in PBS, then permeabilized for 10 minutes with 0.5% Triton X-100.

(3) Methanol/acetone fixation. Cells were fixed in methanol for 5 minutes at -20°C, followed by acetone for 30 seconds at -20°C, and either rinsed in PBS for immediate use or air dried and stored at -20°C.

Double label immunofluorescence was performed essentially as described by Harlow and Lane (1988). Each well of a multiwell slide was preincubated for 20 minutes in blocking buffer (either 20% FCS, 0.5% Tween-20 in PBS or 2% dried skim milk, 0.1% Tween-20 in PBS), treated with primary antibody diluted in blocking buffer for 45 minutes at RT, and washed for 15 minutes. A second primary antibody, raised in a different species from the first, was applied and incubated 45 minutes at RT. After washing, a mixture of fluoro-chrome-labelled secondary antibodies was applied for 45 minutes at RT, and the slides were washed extensively before mounting in 90% glycerol, 50 mM Tris, pH 8.0, containing 1 mg/ml *p*-phenylene diamine to reduce bleaching. Slides were examined on an MRC-600 laser scanning confocal microscope and the images recorded on an optical disc.

Surface/intracellular binding

The method used was modified from that of Johnson et al. (1990). Cells were grown to near confluence in 6-well plates. To estimate the amount of cell surface binding sites, the cells were chilled to 4°C, washed with DPBS+1% BSA, then incubated with ¹²⁵I-labelled antibody in DPBS+1% BSA for two hours at 4°C. After washing, the cells were harvested in 0.1 M NaOH, transferred (together with cell debris) to a Luckham tube and the counts measured in a gamma counter.

To estimate the total number of binding sites, cells in replicate plates were permeabilized before being incubated with ¹²⁵I-labelled antibody. Because washing with detergent leads to the loss of cells from the plate, the cells were first fixed with glutaraldehyde, permeabilized and treated with NaBH₄ (as described above), before being processed in parallel with the non-permeabilized cells.

Endocytosis assays

Endocytosis of the hybrid proteins was detected in transiently transfected COS7 cells essentially by the method of Bretscher and Lutter, 1988. Portions of packed cells (100 µl) were first labelled with 5 mg/ml of the reagent biotin-3, 3'-dithiobis(sulphosuccinimidyl propionate). To measure endocytosis, labelled cells were put into a small volume of DPBS/FCS and split into three equal portions: (1) one was held at 0°C; (2) one was held at 0°C and, in parallel with the third sample, treated with cysteine; (3) the third sample was diluted into 7 volumes of DPBS/FCS at 37°C, held there for 20 minutes with occasional shaking, chilled to 0°C, spun out and resuspended in a small volume of DPBS/FCS at 0°C. Cysteine stripping was performed as described by Bretscher (1992) in a modification of the original reduction method and cell lysates made as described, in 2% NP40. Lysozyme chimeras were immunoprecipitated on 10 µl of Sepharose to which antibody F10 had been coupled. The immunoSepharose was eluted in non-reducing SDS sample buffer at 50°C and analyzed by

lys/MPR



lys/LDLR

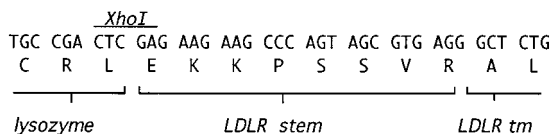


Fig. 1. Sequences at the junctions between lysozyme and receptor domains in the lys/MPR and lys/LDLR chimeras. The last residue of chicken lysozyme is fused either to residue 1271 of the bovine MPR or to residue 760 of the human LDLR. Regions of the MPR and LDLR 'stem' (i.e. residues N-terminal to the transmembrane domain) were included to provide a spacer between lysozyme and the membrane.

electrophoresis on a gradient 5-20% polyacrylamide gel, which was then blotted onto nitrocellulose. The biotin-containing proteins were detected by labelling the blot with ¹²⁵I-streptavidin (Amersham, UK) and the blot was autoradiographed at -70°C on flashed film for 1 to 3 days. Comparison of the label present in gel bands was done directly by counting excised bands in a gamma counter.

RESULTS

Chimeric proteins

A series of chimeras was constructed where the transmembrane and cytoplasmic domains of the MPR were fused to lysozyme, a secretory protein not normally found in COS cells.

A chimeric 'lys/MPR' molecule was created by fusing the transmembrane and cytoplasmic domains of the bovine MPR to the C terminus of chicken lysozyme, leaving 4 residues of the MPR luminal domain to provide a spacer between lysozyme and the membrane. Similarly, a hybrid lysozyme-LDLR molecule (lys/LDLR) was created by replacing most of the human LDLR extracellular domain with lysozyme, leaving an 8 residue spacer followed by the complete transmembrane and cytoplasmic domains of the LDLR (Fig. 1). Additional chimeras were made by exchanging sequences coding for the cytoplasmic domains between lys/MPR and lys/LDLR using a PCR-based strategy, and various truncated or mutant forms of the MPR tail were created by introducing stop codons or point mutations using site-directed mutagenesis. No changes were made to the amino acid sequences (other than those described here) as a result of the cloning procedures.

The various chimeras are shown in Fig. 2. The first four (lys/LDLR, lys/MPR, lys/L/M and lys/M/L) are designed to compare the abilities of the cytoplasmic and transmembrane domains of the MPR and the LDLR to determine the intracellular distribution of lysozyme. The two tyrosines critical for endocytosis (Y²⁴Y²⁶) are replaced with alanine and valine (A²⁴V²⁶) to create the internalization-deficient lys/Δ2Y mutant. Truncation of lys/MPR after residues 1401, 1355, 1333, 1305 or 1301 creates proteins that contain 105, 59, 35, 9 or 5 aa of the 163 aa MPR tail, respectively. However a

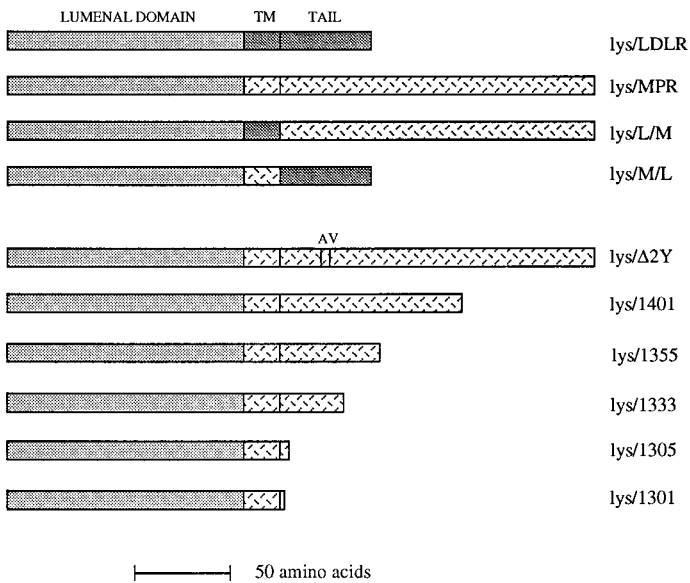


Fig. 2. Schematic representation of the chimeric constructs. The chimeras are shown as rectangles, with the N-termini on the left, and divided into sections corresponding to the luminal, transmembrane (TM) or cytoplasmic (TAIL) domains. Sections are shaded to indicate domains derived from lysozyme (light dots on left), MPR (hatched) or LDLR (darker dots). For chimeras with cytoplasmic truncations, the last residue of the bovine MPR sequence present in the construct is given in the name e.g. lys/1401. The mutations shown in lys/ Δ 2Y convert Y24 to alanine and Y26 to valine.

variant of lys/1333 has since been found that codes for additional residues 36 to 39 and a further repeat of residues 32 to 35 (i.e. 8 further residues) before the expected stop codon.

A SAY1-based plasmid containing the SV40 origin of replication was used to direct the expression of chimeric proteins in COS cells under the control of the adenovirus major late promoter (Munro and Pelham, 1987). This plasmid replicates to high copy number in COS7 cells, a monkey fibroblast line, derived from CV1 cells, which contains an integrated copy of the SV40 T antigen gene, allowing a high level of expression of target sequences (Mellon et al., 1981).

Expression in COS cells

The lys/MPR and lys/LDLR chimeras, when expressed in COS cells and analysed by SDS-PAGE followed by immunoblotting with an anti-lysozyme antiserum, are of the expected size (Fig. 3). Furthermore, an antiserum raised against a fusion protein that contains the bovine MPR cytoplasmic domain (anti-JG2; Glickman et al., 1989) recognizes lys/MPR, but does not recognize the endogenous COS cell MPR or lys/LDLR (Fig. 3). Truncated and mutant forms of lys/MPR also behave as proteins of the expected size and are recognized by both anti-lysozyme and anti-JG2 antibodies (not shown), with the exception of chimeras containing 35 residues or less of the MPR tail. These mutants are not recognized by the anti-JG2 antiserum, possibly because the membrane-proximal portion of the MPR tail is highly conserved between mouse, cow and man.

Differential distribution of lys/MPR and lys/LDLR

The localization of the lys/MPR and lys/LDLR chimeras when

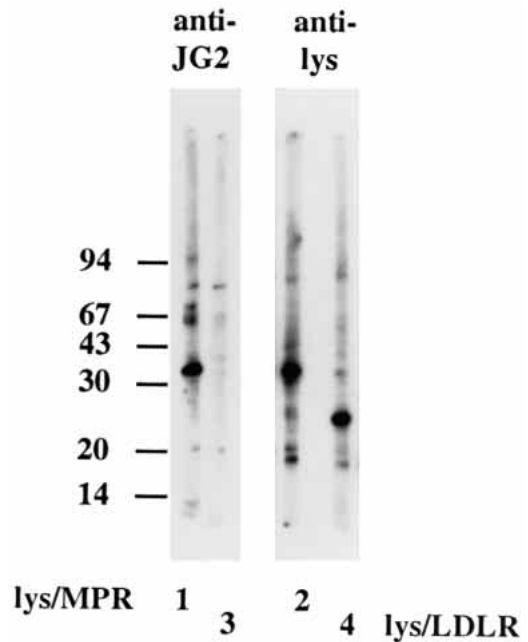


Fig. 3. Expression of chimeric proteins in COS cells. Proteins from COS cells transfected with either lys/MPR or lys/LDLR were separated on a 5–25% SDS-polyacrylamide gel and blotted onto nitrocellulose. Replicate blots were probed with either a rabbit anti-lysozyme antiserum or a rat anti-JG2 antiserum (followed by rabbit anti-rat IgG) and detected with 125 I-Protein A. The sizes of the labelled bands are consistent with the predicted molecular masses of the chimeric proteins. Size standards (kDa) are shown on the left.

expressed in COS cells was examined by indirect immunofluorescence microscopy of methanol/acetone fixed cells. As shown in Fig. 4, lys/MPR is found primarily in an intracellular compartment with a perinuclear, reticulate morphology characteristic of the TGN, as well as in many small vesicles distributed throughout the cytoplasm that are more numerous in highly-expressing cells. In contrast, lys/LDLR shows a diffuse plasma membrane staining similar to that seen when the intact LDLR is expressed in COS cells and stained with anti-LDLR mAb C7. A four hour cycloheximide treatment did not change this distribution (not shown), and lys/MPR staining was the same using antibodies to either the luminal or cytoplasmic domains, indicating that the hybrid protein is intact. Neither chimera is retained in the ER, a common fate of misfolded proteins. Disruption of the MPR tail internalization signal results in a chimeric protein (lys/ Δ 2Y) that is found predominantly at the plasma membrane. This implies that lys/MPR is recycling in the cell and not simply retained in the Golgi, as failure to endocytose has been shown to result in the surface accumulation of MPR that is recycled through the plasma membrane (Lobel et al., 1989).

To confirm that these constructs are actually present on the plasma membrane, the chimeras were tested for their accessibility to mAb F10, which was added at 4°C to the medium of transfected, nonpermeabilised cells. The proportion of chimeric receptor found on the surface was estimated by binding iodinated antibodies in the presence and absence of detergent (Fig. 5). After subtraction of background binding observed on non-transfected cells, only 6% of lys/MPR, but

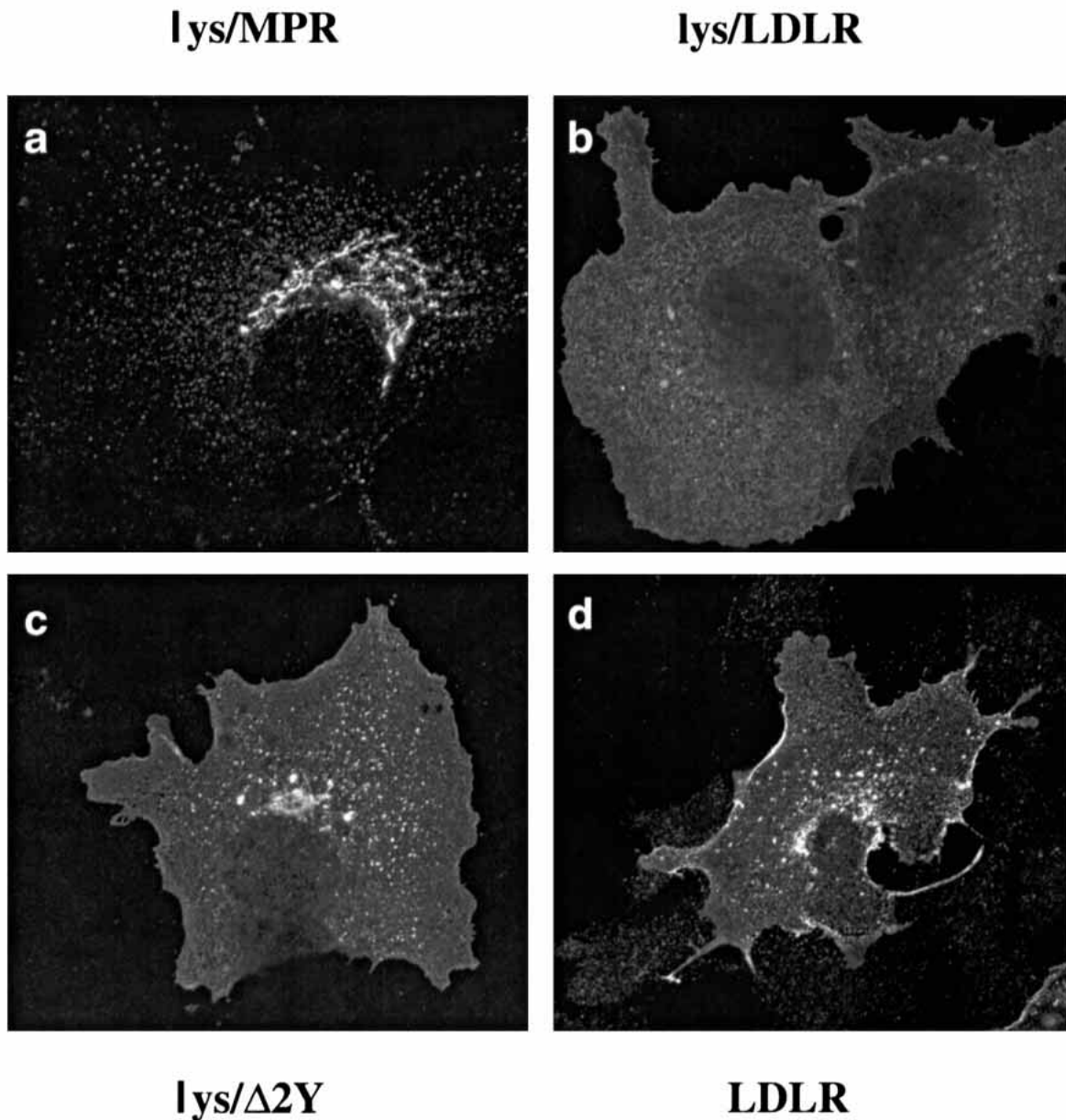


Fig. 4. Immunofluorescent localization of lys/MPR, lys/LDLR, lys/Δ2Y and human LDLR expressed in COS cells. Indirect immunofluorescence of methanol/acetone-fixed cells using either anti-lysozyme mAb D1.3 (a,b,c) or anti-LDLR mAb C7 (d) followed by fluorescein-conjugated second antibody. The lys/MPR chimera is found primarily in intracellular structures rather than on the plasma membrane, whereas labelling of the plasma membrane can be readily seen for lys/LDLR, the internalization-deficient lys/Δ2Y and for the human LDLR.

83% of the lys/LDLR and 75% of the lys/Δ2Y, was located at the plasma membrane.

Fig. 6 shows that the cytoplasmic domains rather than the transmembrane regions determine the distribution of the hybrid molecules. Lys/L/M with the LDLR transmembrane segment has an intracellular localization comparable to that of lys/MPR, while lys/M/L, like lys/LDLR, is found on the surface. Deletion of most of the MPR tail, leaving either 3 or 7 residues to provide a membrane anchor (lys/MPR 1301 and lys/1305, respectively), results in considerable ER retention. Proteins that are not well folded or correctly positioned in the membrane are often retained in the ER (Hurtley and Helenius, 1989), and both lys/1301 and lys/1305 have C-terminal

sequences reminiscent of the 'KKXX' ER retention signal (Nilsson et al., 1989) (ie. lys/1301: KKK; lys/1307: RRXX). Nevertheless, immunofluorescent labelling of transfected cells at 4°C shows that both lys/1301 and lys/1305 are also expressed on the surface (not shown, but see next section for surface labelling of lys/1301).

Thus the MPR cytoplasmic tail specifies the intracellular localization of lys/MPR. All chimeras with truncated MPR tails that retain an intact internalization signal (lys/1333, lys/1355 and lys/1401) are found intracellularly, and their distribution is indistinguishable from those with a full length MPR tail (for an example see Fig. 7 for lys/1333 expression in stably transfected CV1 cells).

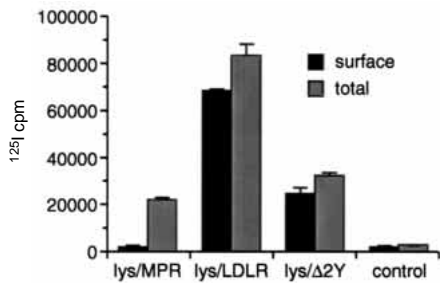


Fig. 5. Cell surface expression of lys/LDLR, lys/MPR and lys/Δ2Y in COS cells. ^{125}I -F10 binding to non-permeabilized (surface) or fixed and permeabilized cells (total) expressing each of the three chimeras, with binding to non-transfected cells serving as a control. The average of triplicate determinations is shown.

Ability of chimeras to be endocytosed

To show that the lysozyme chimeras recycle via the plasma membrane, the capacity of lys/LDLR molecules to undergo endocytosis was observed. Cell surface proteins were labelled with biotin and endocytosed molecules were detected essentially by the method of Bretscher and Lutter (1988). The results are shown in Fig. 8.

Those hybrid molecules that are largely present on the cell surface (lys/LDLR and lys/1301) are readily labelled with biotin-3,3'-dithiobis(sulphosuccinimidyl propionate) as shown in Fig. 8 (lanes d and g). The labelled proteins are extracted from cell extracts by immunoprecipitation with the F10 monoclonal antibody bound to Sepharose, fractionated on an SDS gel, blotted onto nitrocellulose and detected with ^{125}I -streptavidin. A major band of the appropriate molecular size is obvious for both constructs lys/LDLR and lys/1301 (lanes d and g) whereas none is visible in a parallel transfection with no construct (lane a). These labelled bands disappear when the cells are reduced prior to extraction (lanes e and h). However, when the cells are warmed for 20 minutes at 37°C, allowing endocytosis to take place, labelled, internalized membrane proteins are protected from the reduction process. Lys/LDLR is evidently endocytosed efficiently (lane f) and the internal pool, almost certainly saturated after 20 minutes at 37°C (Bretscher and Lutter, 1988), of the lys/LDLR construct is up to about 20%. By this criterion most of the lys/LDLR

molecules are on the cell surface at steady state, in agreement with the estimate from the antibody labelling experiment. Lys/1301 is only marginally protected by warming the cells to 37°C for 20 minutes. Most of the hybrid (90% or more) is essentially trapped on the cell surface as the protein lacks a signal for efficient coated pit endocytosis, though a limited degree of internalization occurs.

Unfortunately, there was too little of hybrids lys/MPR and lys/1333 on the cell surface to label adequately in order to measure endocytosis satisfactorily. To provide evidence of the recycling of lys/MPR, its ability to endocytose F10 antibody at 37°C was tested. Cells transfected with lys/MPR are able to take up and internalize the F10 antibody, bound to lys/MPR (Fig. 9). In contrast, no internalized antibody could be seen in control cells incubated at 4°C (not shown).

Differential distribution of lys/MPR and the endogenous MPR in stable cell lines

The chimeras lys/MPR, lys/1401, lys/1355 and lys/1333 in stably expressing CV1 cell lines all show the same characteristic perinuclear, reticulate staining pattern (see Fig. 7). Little surface staining was evident with these constructs compared to the surface staining in stable lines expressing reasonably high levels of lys/LDLR and lys/Δ2Y (not shown).

Labelling of lys/MPR (and also lys/1333 and lys/1355) shows considerable, but not precise, overlap with galactosyl transferase, an enzyme restricted to the medial Golgi (Fig. 10). This indicates that the constructs recycle to somewhere in the Golgi region, most likely the TGN (Duncan and Kornfeld, 1988; Snider and Rogers, 1985), however the various cisternae and the TGN cannot be resolved by fluorescence microscopy.

In rat hepatoma cells, MPR has been shown by immunoelectron microscopy to be present in coated pits in the TGN but also to be clustered into interior membranes in the prelysosome compartment (Klumperman et al., 1993).

Typically, the MPR is found in the TGN or prelysosomes, although its relative distribution between the various locations along its recycling route varies between cell types (Griffiths et al., 1988). In a bovine fibroblast primary cell line, the MPR is almost entirely confined to the TGN, where it colocalizes with gamma adaptin (Glickman et al., 1989). In contrast (Fig. 11), the endogenous MPR in CV1 cells is seen almost exclusively

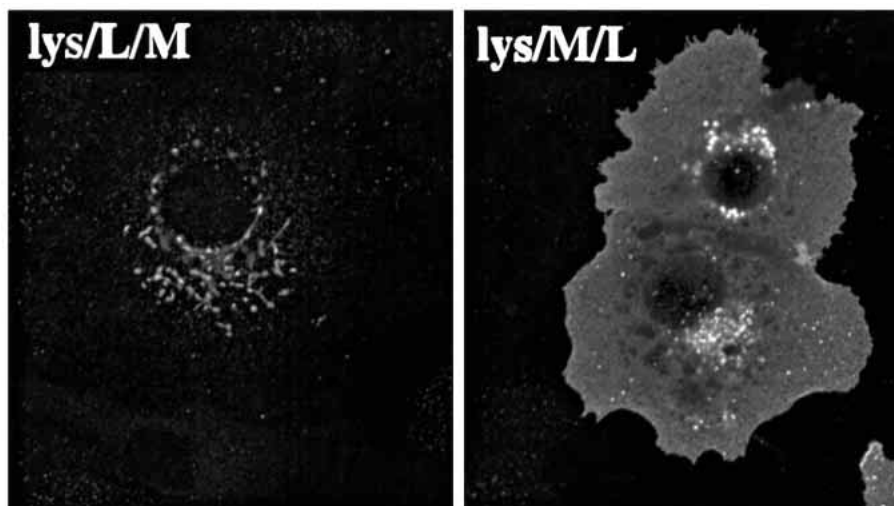


Fig. 6. The cytoplasmic domain of the MPR is required for its intracellular localization. Indirect immunofluorescence of glutaraldehyde-fixed COS cells expressing either lys/L/M (LDLRtm + MPR tail) or lys/M/L (MPR tm + LDLR tail) using mAb F10. The distribution of lys/L/M is similar to that of lys/MPR, whereas lys/M/L has the same distribution as lys/LDLR.

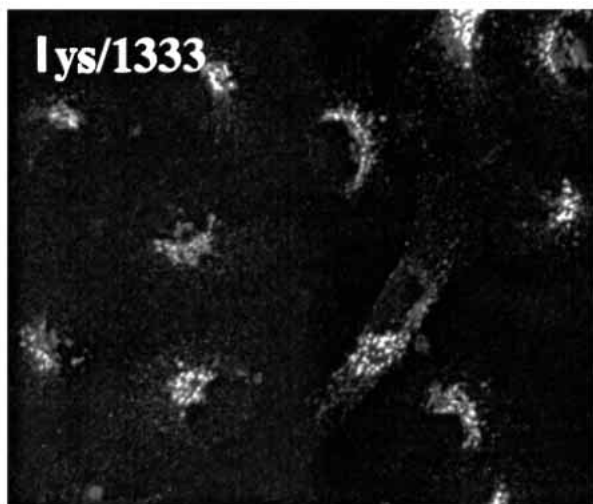


Fig. 7. Indirect immunofluorescence of CV1 cells stably transfected with the lys/1333 construct. Cells were subjected to methanol/acetone fixation and labelled with anti-lysozyme mAb D1.3 and an FITC-conjugated second antibody. A low magnification view of a confluent monolayer is shown to illustrate the degree of homogeneity of expression. Similar patterns of expression were seen for constructs lys/1355 and lys/1401 and lys/MPR.

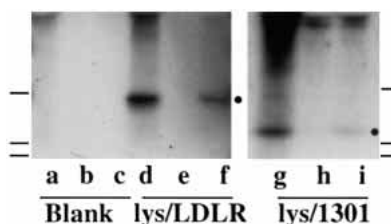


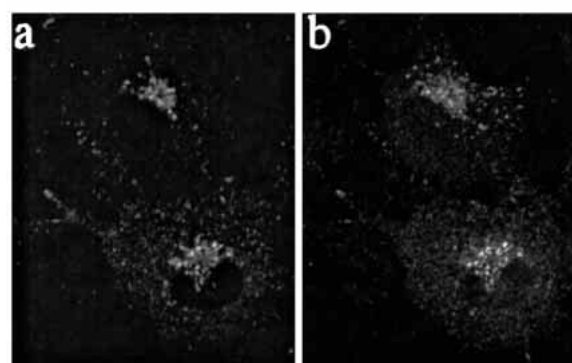
Fig. 8. Endocytosis of hybrid molecules. Autoradiographs showing the indicated lysozyme chimeras from transfected COS cells. The lysozyme hybrids were isolated on an F10 antibody-Sepharose column, from cells labelled at 0°C with biotin, run on an SDS gel, blotted and detected with ^{125}I -streptavidin. The first lane of each set of three samples (a,d and g) represents total isolated labelled material. The second lane of each set (b,e and h) is material from cells labelled and reduced at 0°C. The third lane of each set (c,f and i) shows lysozyme chimera (except c, which was a blank transfection with no construct) from cells labelled at 0°C, incubated at 37°C to allow endocytosis of biotinylated surface material, then reduced at 0°C. Markers are indicated representing molecular masses of 12 kDa, 17 kDa and 30 kDa, respectively.

in vesicular structures, likely to be prelysosomes, which double label with the monoclonal antibody 1B5. This distribution is clearly distinct from that of the lys/MPR chimera and is not affected by the latter's coexpression. Therefore, although the MPR tail is sufficient to specify the intracellular retention of the chimeric protein, the lys/MPR hybrid does not have exactly the same recycling behaviour as the intact MPR in CV1 cells, even though it clearly does recycle as shown by its ability to endocytose bound F10 antibodies (Fig. 9).

DISCUSSION

We have created chimeric proteins consisting of lysozyme, a transmembrane region and the cytoplasmic tail of either MPR

internalized F10 total chimera



lys/MPR

Fig. 9. Antibody uptake by lys/MPR transiently expressed in COS cells. Transfected cells were allowed to endocytose mAb F10 added to the medium (at 25 $\mu\text{g}/\text{ml}$) for 1 hour at 37°C. Internalized antibody was detected by labelling the glutaraldehyde-fixed and permeabilized cells with FITC-conjugated anti-mouse IgG antiserum (shown on the left) and the chimeric proteins were detected by double-labelling with a rabbit anti-lysozyme antiserum (shown on the right).

anti-galactosyl transferase anti-lysozyme

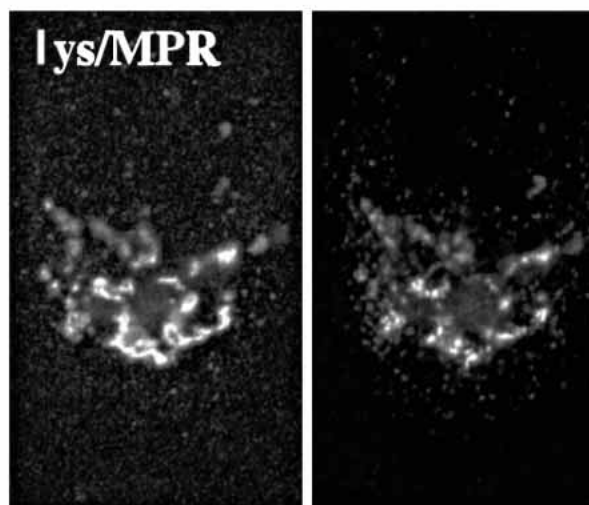


Fig. 10. Intracellular distribution of lys/MPR compared with a Golgi marker by double label immunofluorescence. The two panels show the perinuclear region of a single CV1 cell fixed in methanol/acetone and double labelled with rabbit anti-galactosyl transferase antiserum (on the left) and mAb D1.3 (on the right). The Texas Red- and fluorescein-conjugated secondary antibodies were detected simultaneously using a laser scanning confocal microscope.

(lys/MPR) or LDLR (lys/LDLR). We believe that these hybrid molecules circulate through various membrane compartments in the cell as has been shown for MPR and other endocytosed molecules (Duncan and Kornfeld, 1988; Stoorvogel et al., 1989; Green and Kelly, 1992). We have expressed the chimeric

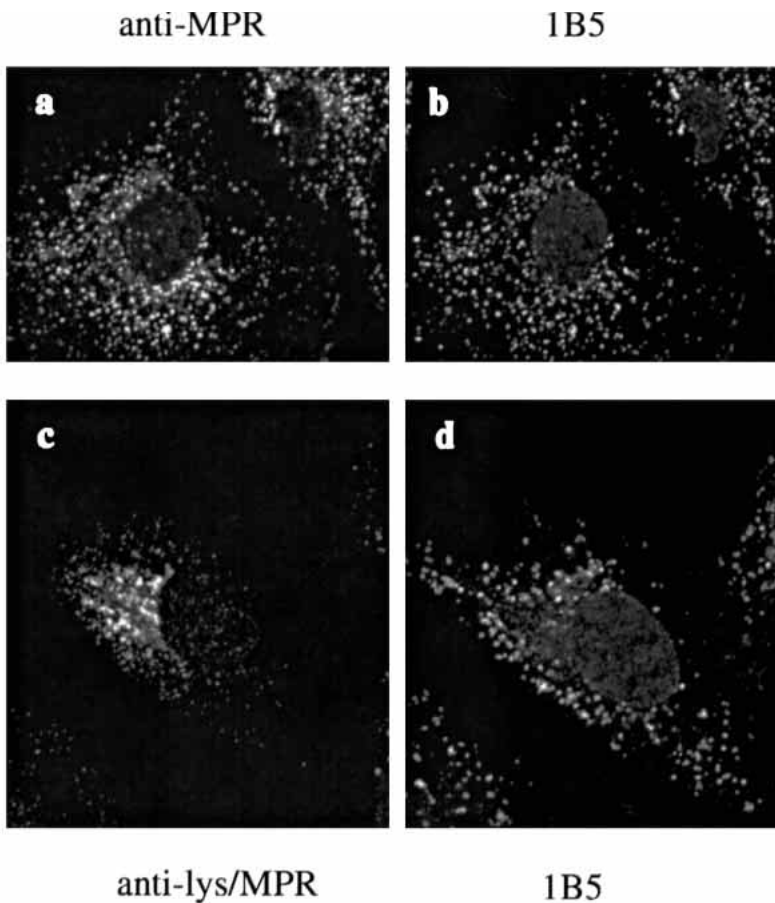


Fig. 11. Comparison of the subcellular distribution of lys/MPR with that of the endogenous MPR. (a and b) In CV1 cells, the endogenous MPR is found in scattered vesicles that can also be labelled with the prelysosomal/lysosomal marker 1B5. In a lys/MPR cell line, the endogenous MPR colocalizes with 1B5 as above. However, anti-bovine MPR tail (anti-JG2) labelling of lys/MPR is clearly distinct from the vesicular staining pattern seen with mAb 1B5 (c and d). We did not try to double label the same cells for MPR and lys/MPR as our antibody against MPR was raised against the whole receptor.

proteins in COS cells or CV1 cells and taken a snapshot view of their steady-state distribution by immunofluorescence microscopy, in order to show the location in which they spend most of their time. We found the lys/MPR hybrid largely in an intracellular compartment in the Golgi region, probably corresponding to the TGN. Dominant signals on lysozyme are not responsible for this distribution, as the lysozyme-LDLR hybrid was found on the cell surface. The membrane-spanning region is known to be important for the Golgi retention of glycosyl transferases (Munro, 1991; Nilsson et al., 1991; Teasdale et al., 1992; Bretscher and Munro, 1993), but exchanging transmembrane domains between these chimeras shows that the MPR tail, and not the transmembrane domain, is responsible for the intracellular distribution of the lys/MPR protein. By expressing truncated forms of lys/MPR, the signal required for intracellular retention was mapped to the first 39 aa of the MPR tail.

The dominant sorting feature identified so far in the membrane proximal third of the MPR tail is the sequence (Y²⁴ KY²⁶ SKV²⁹) apparently containing overlapping but distinct signals for endocytosis by plasma membrane coated pits and lysosomal enzyme sorting mediated by TGN coated pits (Johnson and Kornfeld, 1992). The signal for endocytosis depends chiefly on the spaced hydrophobic residues Y²⁴, Y²⁶ and V²⁹ (Lobel et al., 1989; Jadot et al., 1992) correlating with the observation that the two tyrosine residues are important for the interaction of the plasma membrane adaptor (HA-2) with the MPR tail (Glickman et al., 1989). However, lysosomal enzyme sorting is minimally altered *in vivo* when tyrosines 24

and 26 are substituted with alanines and other features and regions of the tail such as the C-terminal residues LLHV¹⁶³ play a role in this process (Johnson and Kornfeld, 1992). Substitution of the two tyrosines does not appear to affect binding of the Golgi adaptor (HA1) to the MPR tail either, indicating that other regions of the tail are involved in efficient Golgi adaptor binding, likely also to be necessary for lysosomal enzyme sorting *in vivo*. However, the precise nature of Golgi adaptor binding sites has still to be determined.

An interesting parallel can be drawn between the behaviour of lys/MPR and a hybrid protein containing the cytoplasmic tail of TGN38 (Luzio et al., 1990; Bos et al., 1993; Humphrey et al., 1993). In this case also, a short, tyrosine-containing sequence within the cytoplasmic tail is sufficient to confer TGN-localization to a reporter protein Tac. This sequence acts as an efficient endocytosis signal dependent on the usual spacing of hydrophobic residues. However, an R335D mutation in the sequence still allows the mutant to undergo efficient endocytosis but abolishes its ability to localize to the TGN (Humphrey et al., 1993). Presumably this mutation allows a greater proportion of the Tac hybrid to cycle via the plasma membrane thus reducing the amount seen in the TGN at steady state, a situation perhaps closer to that observed with the lys/LDLR construct.

The steady-state localization of the MPR varies between the TGN and prelysosomes in different cell types and may be influenced by the presence of ligand (Griffiths et al., 1988; Brown, 1990). Although the cytoplasmic tail of the MPR promotes the intracellular retention of the receptor, in CV1

cells, the luminal domain is apparently required to allow a greater proportion of the receptor to appear in the prelysosomes rather than the TGN at steady state. Recently, Klumperman et al. (1993) have performed a careful study of MPR distribution in HepG2 and BHK cells by electron microscopy of immunolabelled cryosections. They found that the MPR exited the TGN via the HA-1 adaptor containing coated vesicles. On arrival in prelysosomes, the MPR was relatively enriched in internal vesicles of this morphologically complex compartment. From our observations, the MPR seems to be present in corresponding locations in CV1 cells. The lys/MPR construct is likely to enter Golgi coated pits with the MPR but it could well recycle between the TGN and the prelysosome with altered kinetics, resulting in a different steady-state distribution. The studies of Brown and co-workers (1986) suggest that the MPR is localized to the TGN in the absence of ligand, whereas conditions that prevent the release of bound ligand result in a redistribution of the MPR to the prelysosome. According to this model, the lys/MPR chimera would be expected to be found in the TGN at steady state as it lacks a ligand-binding domain. In fact, lys/MPR behaves similarly to the MPR in I cells, which do not attach the M6P recognition marker to lysosomal enzymes and are therefore devoid of ligand (Brown, 1990). However, a number of studies using reagents that perturb ligand binding have failed to find an effect on surface-to-endosome transport of MPR (Brulke et al., 1987; Pfeffer, 1987). Ligand binding does not appreciably affect the surface to TGN route in K562 human erythroleukemia cells, as treatments that increase (chloroquine, monensin, β -galactosidase) or decrease (M6P, cycloheximide, tunicamycin) receptor occupancy did not affect the resialylation of surface MPR treated with neuraminidase (Jin et al., 1989). Therefore, the overall recycling of MPR is not ligand dependent; nevertheless, it is intriguing to speculate that ligand, perhaps by some form of signal transduction, influences the rate of a transport step and therefore changes the steady-state distribution of the MPR at least in some cell types, including CV1 cells.

Recent studies have provided other examples of receptors that show a ligand-dependent increase in the rate of transport. The accumulation of the EGF-receptor in the multivesicular bodies of prelysosomes seems to depend on kinase activity in response to EGF (Felder et al., 1990). Examples also include the pIg receptor (Bomsel and Mostov, 1991) and the human KDEL receptor (Lewis and Pelham, 1992). If the extent of traffic along a certain pathway is responsive to the amount of ligand in the system, the efficiency of the transport mechanism would be optimized. Clearly, in the case of the MPR, ligand binding affects the conformation of the receptor's cytoplasmic tail, which alters its ability to bind to other molecules, e.g. heterotrimeric G proteins (Murayama et al., 1990) and perhaps clathrin coat proteins in some way, which may in turn affect one or more transport events. In this respect it is interesting that Méresse and Hoflack (1993) have observed that the MPR is phosphorylated on its cytoplasmic tail as it passes through the *trans*-Golgi region.

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