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# The overexpressed human 46-kDa mannose 6-phosphate receptor mediates endocytosis and sorting of β-glucuronidase

(lysosomal enzymes/membrane receptors/cation dependence/amplification)

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**ABSTRACT** We studied the function of the human small (46-kDa) mannose 6-phosphate receptor (SMPR) in transfected mouse L cells that do not express the larger insulin-like growth factor II/mannose 6-phosphate receptor. Cells overexpressing human SMPR were studied for enzyme binding to cell surface receptors, for binding to intracellular receptors in permeabilized cells, and for receptor-mediated endocytosis of recombinant human β-glucuronidase. Specific binding to human SMPR in permeabilized cells showed a pH optimum between pH 6.0 and pH 6.5. Binding was significant in the presence of EDTA but was enhanced by added divalent cations. Up to 2.3% of the total functional receptor could be detected on the cell surface by enzyme binding. We present experiments showing that at very high levels of overexpression, and at pH 6.5, human SMPR mediated the endocytosis of β-glucuronidase. At pH 7.5, the rate of endocytosis was only 14% the rate seen at pH 6.5. Cells overexpressing human SMPR also showed reduced secretion of newly synthesized β-glucuronidase when compared to cells transfected with vector only, suggesting that overexpressed human SMPR can participate in sorting of newly synthesized β-glucuronidase and partially correct the sorting defect in mouse L cells that do not express the insulin-like growth factor II/mannose 6-phosphate receptor.

Acid hydrolases (lysosomal enzymes) are synthesized and glycosylated in the endoplasmic reticulum. After modification in the Golgi apparatus, they acquire the mannose 6-phosphate recognition marker (1–5). Most of the newly synthesized acid hydrolases are transported from the trans-Golgi network to lysosomes by receptors that recognize mannose 6-phosphate residues (6). Some of the acid hydrolases are secreted, and some are recaptured by receptor-mediated endocytosis.

Two mannose 6-phosphate receptors have been identified. The first described was the "215-kDa receptor" (7), which, when cloned, was found to be much larger (8–10) and found to be a bifunctional receptor that also binds and mediates endocytosis of insulin-like growth factor II (IGF-II) (11–13). This receptor is referred to here as the IGF-IIR/MPR, though it has also been referred to as the large MPR, the 270-kDa MPR, the 300-kDa MPR, the cation-independent MPR, and the IGF-II receptor in various contexts. The 46-kDa receptor, which was subsequently discovered and purified from bovine liver, required divalent cations for optimum binding and was named the cation-dependent MPR (14, 15). However, it was recently reported to bind ligand in the absence of added cation (16). The purified human 46-kDa receptor did not require divalent cations for binding (17). Thus, use of the term human 46-kDa MPR seems more appropriate for the human small receptor. In this paper, we will abbreviate this to HSMPR for human small mannose 6-phosphate receptor.

Both the human large receptor (HIGF-IIR/MPR) and the human small receptor (HSMPR) have been cloned and have been expressed in mammalian cells (8–10, 18, 19). The IGF-IIR/MPR has been studied more extensively, and several lines of evidence suggest that it is the more important receptor for sorting acid hydrolases to lysosomes. (*i*) Antibody to the IGF-IIR/MPR blocked endocytosis and sorting (20–22). (*ii*) Mouse cells not expressing the IGF-IIR/MPR secreted most of their newly synthesized lysosomal enzymes (14, 23, 24). (*iii*) The cloned IGF-IIR/MPR cDNA, on transfection of IGF-IIR/MPR-negative cell lines, fully corrected their sorting defect and also led to receptor-mediated endocytosis of exogenous ligands (acid hydrolases and IGF-II) (13, 25, 26).

The role of the HSMPR in the transport of mannose 6-phosphate-containing ligands is much less clear. Stein *et al.* (27) showed that after addition of anti-HSMPR antibody to IGF-IIR/MPR-deficient Morris hepatoma 7777 cells, secretion of newly synthesized lysosomal enzymes increased. This was taken as evidence that the rat SMPR participated in sorting acid hydrolases to lysosomes. Stein *et al.* (27) also showed that adding the anti-HSMPR antibody to human fibroblasts or Hep G2 hepatoma cells, which express both types of receptors, did not impair sorting of lysosomal enzymes. Sorting was inhibited only when both anti-HIGF-IIR/MPR antibody and anti-HSMPR antibody were added to the medium. This result suggested that sorting by the SMPR could only be demonstrated in the absence of the IGF-IIR/MPR (22).

We have transfected the HSMPR cDNA into mouse L cells that are defective for the IGF-IIR/MPR (23). The vector used, pMSXND, contains the dihydrofolate reductase gene, which enables the subcloned cDNA to be coamplified with that gene and overexpressed by selection for growth in the presence of methotrexate (25). Cell lines that express only endogenous murine SMPR and cell lines overexpressing several different levels of HSMPR were obtained. We studied enzyme binding to the overexpressed HSMPR in permeabilized cells. We also studied the ability of the overexpressed HSMPR to mediate endocytosis and the ability of the overexpressed receptor to correct the sorting defect of IGF-IIR/MPR-deficient cells. The ligand used for binding and endocytosis studies was recombinant human β-glucuronidase produced in the mouse L-cell overexpression system described above, and purified from secretions.

## MATERIALS AND METHODS

**Expression of HSMPR.** The HSMPR cDNA was kindly provided by Regina Pohlmann and Kurt von Figura (18). The

Abbreviations: SMPR, small (46-kDa) mannose 6-phosphate receptor; HSMPR, human SMPR; IGF-IIR/MPR, insulin-like growth factor/mannose 6-phosphate receptor; HIGF-IIR/MPR, human IGF-IIR/MPR.

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eukaryotic expression vector pMSXND was a generous gift of Se-Jin Lee and Daniel Nathans (25, 28). IGF-IIR/MPR-negative mouse L cells were kindly provided by Stuart Kornfeld (23). The cDNA of HIGF-IIR/MPR (9) or HSMPR (18) was inserted into the *Xho* I cloning site of pMSXND, which contains the genes for mouse dihydrofolate reductase and for resistance to G418 (28). Receptor-negative mouse L cells were transfected by a calcium phosphate transfection. Stable transformants were selected for resistance to G418 (400 $\mu$g/ml), and positive cell lines were further selected for amplification with 0.2 $\mu$M and 3.2 $\mu$M methotrexate in Dulbecco's modified Eagle's medium (DMEM) with 5% dialyzed fetal bovine serum as described (13, 25).

**Metabolic Labeling and Binding to Mannose 6-Phosphate Affinity Column.** The cells were labeled metabolically with [$^{35}$S]methionine/[$^{35}$S]cysteine (Tran$^{35}$S-label, ICN) essentially as described (25). Membranes were prepared from $^{35}$S-labeled cells and washed as described (25) except that EDTA was not present, and phenylmethylsulfonyl fluoride and iodoacetamide were added to the other protease inhibitors. Each membrane pellet was solubilized in 2 ml of 50 mM Mes, pH 6.5/150 mM NaCl/1% (vol/vol) Triton X-100/0.1% sodium deoxycholate with leupeptin (10 $\mu$g/ml), pepstatin (10 $\mu$g/ml), aprotinin (0.17 trypsin inhibitor unit/ml), phenylmethylsulfonyl fluoride (1 mM), and iodoacetamide (5 mM) overnight on ice. Undissolved material was removed by ultracentrifugation at 100,000 × *g* for 30 min at 4°C. The supernatant was adjusted to 5 mM $\beta$-glycerophosphate and 10 mM $MnCl_2$ (final concentrations), and each sample was mixed with 2 ml of phosphomannan-Affi-Gel 15 affinity resin (15) that was preequilibrated with the solubilization buffer. The mixtures were incubated at 4°C for 2 hr on a rotating wheel. Resin was collected by centrifugation at 600 × *g*, loaded into small columns, and washed with 10 column volumes of buffer A (50 mM Mes, pH 6.5/150 mM NaCl/0.05% Triton X-100/5 mM $\beta$-glycerophosphate) containing 10 mM $MnCl_2$. Resin was transferred to tubes for batch elution with 1.5 ml of buffer A/10 mM $MnCl_2$/5 mM glucose 6-phosphate followed by 1.5 ml of buffer A/10 mM mannose 6-phosphate. Aliquots of eluted material were analyzed by SDS/PAGE (29).

**Enzyme Binding to Permeabilized Overexpressing Cells.** Overexpressing cells were grown as a monolayer in 24-well cluster dishes. For binding assays, cells were permeabilized with 0.25% saponin in 50 mM Hepes, pH 7.0/150 mM NaCl/5 mM $\beta$-glycerophosphate/0.5% human serum albumin/10 mM mannose 6-phosphate (0.5 ml per well) for 30 min on ice. The cells were then washed three times with 0.15 M NaCl/0.05% saponin. They were incubated with recombinant human $\beta$-glucuronidase (5000–90,000 units/ml) in 50 mM Mes, pH 6.3/150 mM NaCl/5 mM $\beta$-glycerophosphate/0.5% human serum albumin/0.5% saponin/10 mM $MnCl_2$ with or without 10 mM mannose 6-phosphate for 20 hr on ice. The cells were then washed five times with phosphate-buffered saline (PBS) containing 0.05% saponin and were solubilized in 1% sodium deoxycholate. The solubilized cells were assayed for $\beta$-glucuronidase (30) and protein (31).

**Endocytosis by Overexpressing Cell Lines.** Overexpressing cells were grown in monolayers in 35-mm dishes. For endocytosis experiments, cells were fed with 40,000 units of recombinant $\beta$-glucuronidase plus 0 or 2 mM mannose 6-phosphate in 1 ml of DMEM containing 1 mM $NaHCO_3$, 0.5% human serum albumin, and 25 mM Mes (pH 6.5) or 25 mM Hepes (pH 7.5) and were incubated in a 37°C incubator without added $CO_2$ for 1 hr. Endocytosis was stopped by washing five times with cold PBS on ice. Cell surface-bound enzyme was eluted by incubation with PBS plus 10 mM mannose 6-phosphate on ice for 30 min. The cells were solubilized in 1% sodium deoxycholate and assayed for internalized $\beta$-glucuronidase and for protein.

**Secretion of Newly Synthesized $\beta$-Glucuronidase.** Cells were split into 35-mm dishes 24 hr before the start of the experiment. At 0 hr, cells were washed and 1 ml of fresh medium with 2 mM mannose 6-phosphate was added. Replicate plates were removed for assay of intracellular $\beta$-glucuronidase at 0 hr. After a 24-hr incubation at 37°C, the medium and cell lysates in 0.5% sodium deoxycholate were assayed for $\beta$-glucuronidase. The amount of newly synthesized $\beta$-glucuronidase was calculated by subtracting the total $\beta$-glucuronidase activity in the cells at 0 hr from the sum of total $\beta$-glucuronidase activity in the cells and in the medium after 24 hr. Percent secretion was calculated from the total $\beta$-glucuronidase activity in the medium divided by the total newly synthesized $\beta$-glucuronidase activity.

## RESULTS

**Metabolic Labeling and Column Binding of Overexpressed HSMPR.** IGF-IIR/MPR-negative mouse L cells selected for overexpression of HSMPR were labeled metabolically, solubilized, and applied to a mannose 6-phosphate affinity column (Fig. 1). The wash with glucose 6-phosphate did not release radioactivity, but the succeeding mannose 6-phosphate wash eluted the retained HSMPR, which showed microheterogeneity, migrating as a broad band from 40 to 46 kDa on SDS/PAGE. The endogenous murine SMPR in the mouse L cells that were transfected with vector only and selected in the same way was not detectable on this exposure.

**Kinetics of Enzyme Binding to Permeabilized Overexpressing Cells.** The cells expressing HSMPR showed mannose 6-phosphate-inhibitable binding of recombinant human $\beta$-glucuronidase (produced from IGF-IIR/MPR-negative mouse L cells overexpressing human $\beta$-glucuronidase) after permeabilization with saponin. Fig. 2*A* shows the effect of enzyme concentration on mannose 6-phosphate-specific enzyme binding to permeabilized cells. Binding saturated at 1.5 $\mu$g of enzyme bound per mg of cell protein. The $K_d$ was estimated to be $2.8 \times 10^{-10}$ M. Fig. 2*B* shows the time course of binding to permeabilized cells. Binding reached equilibrium in 8 hr.

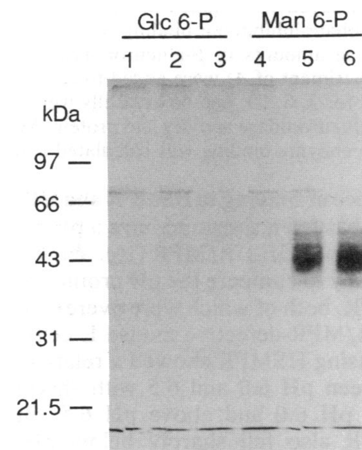


FIG. 1. SDS/PAGE of metabolically labeled HSMPR. Cells were metabolically labeled with Tran$^{35}$S-label. Membranes were solubilized and mixed with phosphomannan-Affi-Gel 15 resin. Aliquots of material eluted with buffer containing 5 mM glucose 6-phosphate (lanes 1–3) or 10 mM mannose 6-phosphate (lanes 4–6) were subjected to SDS/PAGE. The gel was prepared for fluorography and the film was exposed overnight. Lanes 1 and 4, cells transfected with the vector without HSMPR cDNA and amplified with 0.2 $\mu$M methotrexate; lanes 2 and 5, cells transfected with the vector with HSMPR cDNA and amplified with 0.2 $\mu$M methotrexate; lanes 3 and 6, cells transfected with the vector with HSMPR cDNA and amplified with 3.2 $\mu$M methotrexate.

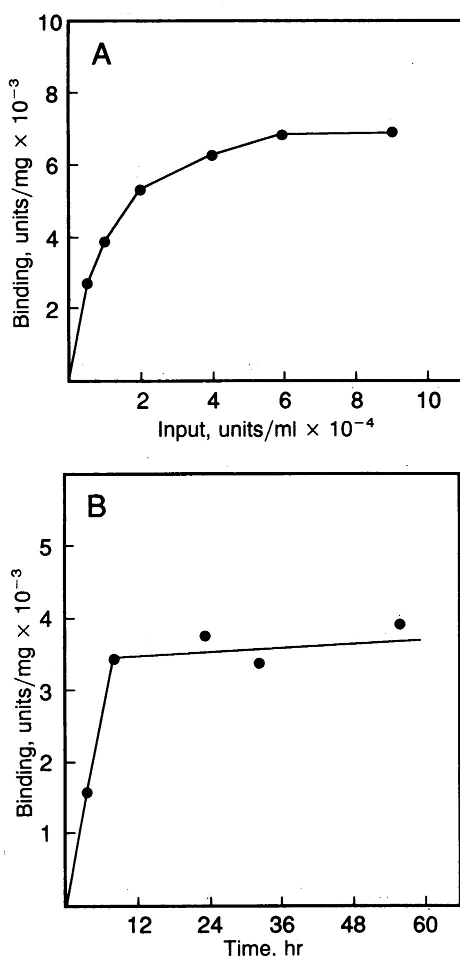


FIG. 2. Concentration dependence and time course of binding of recombinant $\beta$-glucuronidase to permeabilized cells overexpressing HSMPR. (*A*) Various amounts of human $\beta$-glucuronidase (5000–90,000 units/ml) were added to each set of wells with or without 10 mM mannose 6-phosphate and incubated at 4°C for 16 hr. Cells were solubilized and assayed for $\beta$-glucuronidase activity and protein. Mannose 6-phosphate-inhibitable binding was calculated and plotted. (*B*) Saturating amounts of $\beta$-glucuronidase (60,000 units/ml, derived from experiment of *A*) were added to each set of wells and incubated at 4°C for 3, 8, 23, and 56 hr. Cells were solubilized and measured for $\beta$-glucuronidase activity and protein. Mannose 6-phosphate-inhibitable enzyme binding was calculated and plotted.

**pH Dependence of Binding to HSMPR and HIGF-IIR/MPR.** Bovine SMPR has been shown to have a pH profile different from that of bovine IGF-IIR/MPR (16). We used the permeabilized cell assay to compare the pH profiles of HSMPR and HIGF-IIR/MPR, both of which were overexpressed in transfected IGF-IIR/MPR-defective mouse L cells (Fig. 3). The cell line expressing HSMPR showed a relatively narrow pH optimum between pH 6.0 and 6.5 with sharply decreasing binding below pH 6.0 and above pH 6.5. Binding to the HIGF-IIR/MPR also fell sharply below pH 6.0, but, in contrast to the HSMPR, it was not decreased when the pH was raised above 6.5. Particularly significant is the marked difference in binding of the two receptors at pH 7.5—where the binding activity of HIGF-IIR/MPR is still maximal and that of HSMPR is only 28% of the binding seen at pH 6.5.

**Cation Dependence of Binding to HSMPR.** Bovine SMPR was originally described as the cation-dependent mannose 6-phosphate receptor because of dependence of ligand binding on divalent cations (14, 15), but the purified HSMPR did not show this dependence (17). We examined the effect of divalent cations on enzyme binding to the HSMPR in permeabilized cells. With (Fig. 4*A*) or without (Fig. 4*B*) EDTA

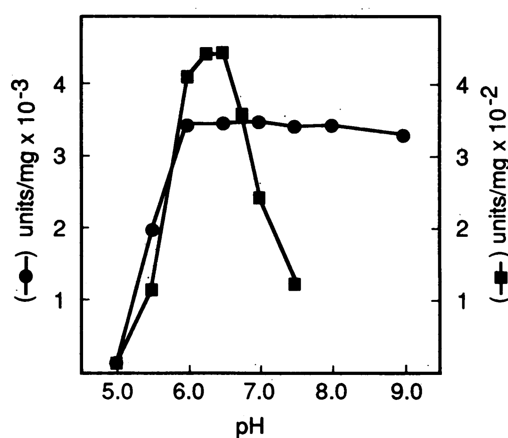


FIG. 3. pH dependence of $\beta$-glucuronidase binding to permeabilized cells overexpressing HSMPR (■) or HIGF-IIR/MPR (●). Cells were prepared and permeabilized with the medium containing 0.25% saponin. Binding media, differing in pH and containing $\beta$-glucuronidase (10,000 units/ml), were added to the wells. Binding to HSMPR was measured at pH 5.0–9.0. Buffer was Mes (pH 5.0–6.5), Hepes (pH 6.75–8.0), or Tris/HCl (pH 9.0). Cells were solubilized and assayed for $\beta$-glucuronidase activity and protein. Mannose 6-phosphate-inhibitable binding activity was calculated and plotted.

in the washing and binding buffers, $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ showed some stimulation. However, the substantial binding in the presence of EDTA and the absence of added cation (Fig. 4*B*) indicates that the HSMPR, though cation-stimulated, is not strictly cation-dependent for binding. Among the three cations studied, $Ca^{2+}$ gave the greatest stimulation, and the maximum stimulation was seen at 2 mM $Ca^{2+}$. The magnitude of stimulation over many experiments ranged from 0% to 54%, and averaged 20%.

**Fraction of Functionally Active HSMPR on the Cell Surface.** Both SMPR and IGF-IIR/MPR are known to be located intracellularly and on the cell surface (30, 32). Table 1 compares the enzyme binding activity on the cell surface with that found intracellularly in cells overexpressing HSMPR or HIGF-IIR/MPR. In the cells expressing HIGF-IIR/MPR, 8.2% of the total enzyme binding in permeabilized cells was on the cell surface—which is near the distribution seen in normal human fibroblasts (30). The cells expressing HSMPR gave lower values for the fraction of receptor on the cell surface (1.2–2.3%), which agrees with a prior estimate of SMPR expression on the surface of U-937, a human monocyte cell line (32).

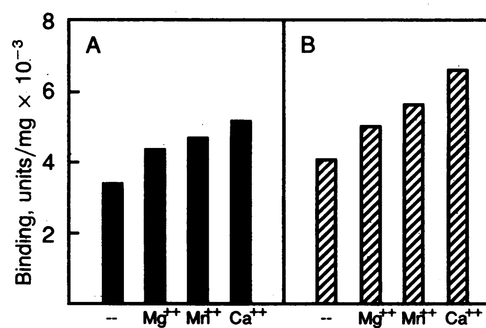


FIG. 4. Effect of divalent cations on enzyme binding to the HSMPR in permeabilized overexpressing cells. Cells overexpressing HSMPR were prepared and washed with permeabilization buffer without (*A*) or with (*B*) 2 mM EDTA. $\beta$-Glucuronidase (10,000 units/ml) was added and incubated 16 hr at 4°C in the absence or presence of 10 mM $Mg^{2+}$, $Mn^{2+}$, or $Ca^{2+}$, without (*A*) or with (*B*) 2 mM EDTA in the buffer. After 16 hr the cells were solubilized, and assayed for enzyme and protein. Mannose 6-phosphate-inhibitable enzyme binding was calculated and plotted.

Table 1. Cell surface and permeabilized binding to cells overexpressing HSMPR or IGF-IIR/MPR

| Cell line* | Mannose 6-phosphate-specific β-glucuronidase binding, units/mg of protein | | % cell surface binding |
|---|---|---|---|
| | Permeabilized | Cell surface | |
| MS9-II, 3.2 | 11,920 | 976 | 8.2 |
| MS-C, 0.2 | 151 | 3.5 | 2.3 |
| SR4C, 0.2 | 2,967 | 35 | 1.2 |
| SR2-1, 0.2 | 13,345 | 269 | 2.0 |
| SR2-1, 3.2 | 12,729 | 299 | 2.3 |

Cells overexpressing the HSMPR cDNA, the HIGF-IIR/MPR cDNA, or vector only were prepared in 24-well cluster dishes. For permeabilized binding, cells were permeabilized with saponin (0.25%) and incubated with saturating amounts of β-glucuronidase (60,000 units/ml) at 4°C for 16 hr, after which the mannose 6-phosphate-inhibitable binding activity was calculated. For cell surface binding, cells were first incubated with wash buffer (50 mM Hepes, pH 7.0/150 mM NaCl/5 mM β-glycerophosphate/0.5% human serum albumin/10 mM mannose 6-phosphate) at 4°C for 30 min. Cells were washed three times with saline, and binding buffer (50 mM Mes, pH 6.3/150 mM NaCl/5 mM β-glycerophosphate/0.5% human serum albumin/10 mM $MnCl_2$, with or without 10 mM mannose 6-phosphate) containing β-glucuronidase (60,000 units/ml) was added and incubated at 4°C for 20 hr. Mannose 6-phosphate-inhibitable binding activity was calculated.

*MS9-II, 3.2: cells transfected with the vector with HIGF-IIR/MPR cDNA and amplified with 3.2 μM methotrexate. MS-C, 0.2: cells transfected with vector only and amplified with 0.2 μM methotrexate. SR4C, 0.2 and SR2-1, 0.2: cells transfected with the vector with HSMPR cDNA and amplified with 0.2 μM methotrexate. SR2-1, 3.2: cells transfected with the vector with HSMPR cDNA and amplified with 3.2 μM methotrexate.

**Endocytosis of Enzyme by HSMPR in Mouse L Cells.** Endocytosis of β-glucuronidase was compared in cells overexpressing either HSMPR or HIGF-IIR/MPR (Table 2). Cells transfected with vector only (i.e., expressing only the endogenous level of murine SMPR and no IGF-IIR/MPR) showed no detectable mannose 6-phosphate-inhibitable endocytosis at either pH 6.5 or pH 7.5. The HSMPR-expressing cells (which had 84 times the binding activity of cells transfected only with vector in the permeabilized binding assay) showed a quite significant rate of mannose 6-phosphate-inhibitable endocytosis, even though the rate was lower than that of the HIGF-IIR/MPR-transfected cells. In contrast to the endocytosis by the IGF-IIR/MPR, endocytosis mediated by the HSMPR was dramatically influenced by pH. The rate of endocytosis at pH 7.5 was only 14% the rate seen at pH 6.5.

Table 2. Endocytosis of β-glucuronidase by cells overexpressing HSMPR

| Cell line* | Mannose 6-phosphate-specific uptake, units/mg of protein | |
|---|---|---|
| | pH 6.5 | pH 7.5 |
| MS-C, 3.2 | 1.25 | 1.3 |
| SR2-1, 3.2 | 453 | 65 |
| SR2-2, 3.2 | 386 | 64 |
| MS9-II, 3.2 | 4518 | 4503 |

Cells were incubated with enzyme (40,000 units/ml) in medium containing 1 mM $NaHCO_3$, buffered with 25 mM Mes (pH 6.5) or 25 mM Hepes (pH 7.5) for 1 hr at 37°C without added $CO_2$ in the presence or absence of 10 mM mannose 6-phosphate. After incubation, cell surface-bound ligand was displaced by incubation at 4°C in phosphate-buffered saline containing 10 mM mannose 6-phosphate. Intracellular β-glucuronidase was measured following solubilization, and mannose 6-phosphate-inhibitable endocytosis was calculated. β-Glucuronidase binding (to permeabilized cells) at pH 6.3 for these cell lines was 12,729 units/mg for SR2-1, 3.2; 11,238 units/mg for SR2-2, 3.2; and 11,920 units/mg for MS9-II, 3.2.

*See footnote to Table 1.

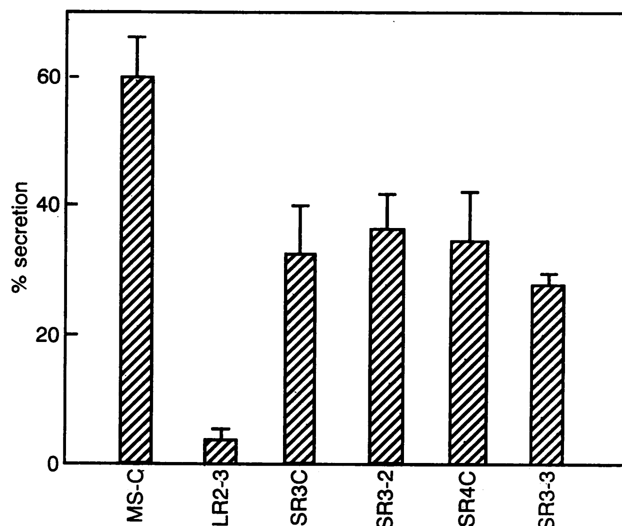


FIG. 5. Effects of expression of HSMPR and HIGF-IIR/MPR on secretion of newly synthesized β-glucuronidase. Intracellular β-glucuronidase activity was measured at 0 hr and after 24 hr of incubation in the presence of 2 mM mannose 6-phosphate. Percent secretion was calculated by determining the fraction of newly synthesized β-glucuronidase activity in the medium after 24 hr of incubation. Cell lines: MS-C, transfected with vector only; LR 2-3, transfected with the vector containing IGF-IIR/MPR cDNA; SR-3C, SR 3-2, SR 4-C, and SR 3-3, transfected with the vector containing HSMPR cDNA. All cell lines were amplified with 0.2 μM methotrexate. Compared with the IGF-IIR/MPR-deficient parental mouse L-cell line MS-C, SR-3C has 20 times, SR 3-2 has 10 times, SR-4C has 6 times, SR 3-3 has 5 times more permeabilized-cell binding activity at pH 6.5.

**Intracellular Sorting by Overexpressed HSMPR.** Mouse L cells expressing only the endogenous murine SMPR secrete a large fraction of their newly synthesized acid hydrolases, showing the importance of the IGF-IIR/MPR in sorting enzyme to lysosomes in L cells. Transfected cells overexpressing either human or bovine IGF-IIR/MPR are largely corrected (25, 26). We examined cells expressing 5–20 times the normal endogenous level of HSMPR to see whether overexpression of the receptor could reduce the level of enzyme secretion and increase the fraction sorted to lysosomes (Fig. 5). In this experiment, cells transfected with vector alone secreted 60% of newly synthesized acid hydrolases. Cells expressing HIGF-IIR/MPR at only 40% the level of murine IGF-IIR/MPR seen in wild-type mouse L cells were fully corrected, secreting only 3% of newly synthesized β-glucuronidase. Cells overexpressing the HSMPR to 5–20 times the level of the endogenous murine receptor showed an intermediate level of secretion, indicating that expression of 5–20 times the endogenous level of SMPR partially corrects the defective enzyme sorting. These corrective effects of the overexpressed HSMPR were inhibited by adding 15 mM $NH_4Cl$ to the culture medium (data not shown).

## DISCUSSION

We describe here an overexpression system for studying the HSMPR in the absence of the IGF-IIR/MPR, and a method for studying the binding of physiological ligands to this receptor in permeabilized cells (16, 27, 32). The range of expression of the HSMPR in the different transfected cell lines was 5–80 times the level of the endogenous murine SMPR in the parental cell line. It was hoped that by eliminating the background of the usually dominant IGF-IIR/MPR activity and raising the signal of the HSMPR by

overexpression, we could shed additional light on its physiological role in lysosomal enzyme transport.

The properties of the overexpressed HSMPR in mouse L cells, as disclosed by studies of ligand binding in permeabilized cells, were generally similar to those observed for the purified bovine and human receptors. The HSMPR in permeabilized cells had a narrow pH optimum for binding recombinant β-glucuronidase (pH 6.0–6.5) with binding falling sharply below pH 6.0 and above pH 6.5. The narrow pH optimum for binding would be expected to allow ligand binding in the Golgi compartments and early endosomes and to favor ligand release in more acidic, late endosomal compartments, as well as at the cell surface, where the pH would be over 7.0. The decrease in binding above pH 6.5 was not seen in the permeabilized cells expressing HIGF-IIR/MPR. The differences in pH optimum between the two receptors could largely explain why both could participate in binding and sorting newly synthesized enzymes in the Golgi apparatus and endosomes while only IGF-IIR/MPR would mediate endocytosis of extracellular enzyme under physiological conditions.

The ability of the overexpressed HSMPR to bind newly synthesized acid hydrolases and effect their sorting to lysosomes was suggested by the decreased secretion of newly synthesized β-glucuronidase by the overexpressing cell lines. However, the HSMPR appeared to be considerably less efficient in intracellular sorting of β-glucuronidase than the expressed HIGF-IIR/MPR, since the latter fully corrected the sorting defect of mouse cells lacking the IGF-IIR/MPR, even at low levels of expression (half the level of IGF-IIR/MPR expressed in wild-type mouse L cells), whereas HSMPR expression 20-fold higher than that of the endogenous murine SMPR only partially corrected the sorting defect in this cell line.

The results presented here indicate that the overexpressed HSMPR can also mediate endocytosis. This was not reported previously for the bovine, human, or murine SMPR. The pH optimum of the HSMPR could partially explain why endocytosis was not seen previously, as the endocytosis was clearly pH-dependent. The endocytosis seen at pH 7.5 was only 14% that seen at pH 6.5. However, pH optimum was not the only factor influencing the ability of the HSMPR to mediate endocytosis. The level of expression was also important. Endocytosis appeared to require a minimum threshold of overexpression, and significant endocytosis was seen only in cells expressing 50–80 times the level of endogenous murine receptor. Transfected cell lines expressing 5–20 times the endogenous level of SMPR showed either nondetectable or barely detectable levels of endocytosis, even though this level of overexpression had a significant effect on sorting.

Waheed *et al.* (33) recently demonstrated that the tetrameric form of the HSMPR bound mannose 6-phosphate ligands with greater affinity than dimeric receptors and that formation of the tetrameric form was favored by ligand binding, neutral pH, and increasing receptor concentration. They suggested that ligand- and pH-induced changes in the quaternary structure of the SMPR might influence and possibly regulate the function of the receptor. Interpreted in this light, the very high levels of HSMPR expression required for detectable endocytosis may reflect the concentration of HSMPR needed to favor tetramer formation on the cell surface. That much lower levels of expression are required to observe a corrective effect on sorting could indicate either that (*i*) tetramer formation is not required for the HSMPR to participate in sorting or (*ii*) it is easier to achieve the high concentration required for tetramer formation in the smaller sorting compartments than to achieve this concentration of HSMPR at the cell surface. In fact, we observed that only 1–3% of the overexpressed HSMPR was present on the cell surface of the mouse L cells. It seems probable, therefore, that the concentration of HSMPR in Golgi and endosomal membranes was considerably higher than the concentrations on the cell surface.

Our findings clearly support the suggestion of Stein *et al.* (27) that the HSMPR can participate in sorting of newly synthesized acid hydrolases. We suggest that failure to see endocytosis by this receptor under physiological conditions relates to three factors: (*i*) the cell surface receptors are not normally exposed to ligand at the optimum pH for binding, (*ii*) they are not normally exposed to saturating concentrations of the mannose 6-phosphate ligands, and (*iii*) they do not normally achieve the high concentration on the cell surface that the experiments presented here suggest is required for mediating endocytosis.

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