

# The Cytoplasmic Tail of the Mannose 6-Phosphate/Insulin-like Growth Factor-II Receptor Has Two Signals for Lysosomal Enzyme Sorting in the Golgi

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**Abstract.** The mannose 6-phosphate/insulin-like growth factor-II (Man-6-P/IGF-II) receptor is known to cycle between the Golgi, endosomes, and the plasma membrane. In the Golgi the receptor binds newly synthesized lysosomal enzymes and transports them directly to an endosomal (prelysosomal) compartment without traversing the plasma membrane. Deletion of the carboxyl-terminal Leu-Leu-His-Val residues of the 163 amino acid cytoplasmic tail of the bovine Man-6-P/IGF-II receptor partially impaired this function, resulting in the diversion of a portion of the receptor-ligand complexes to the cell surface, where they were endocytosed. The same phenotype was observed when 134 residues of the cytoplasmic tail were

deleted from the carboxyl terminus. Disruption of the Tyr<sup>24</sup>-Lys-Tyr-Ser-Lys-Val<sup>29</sup> plasma membrane internalization signal alone had little effect on Golgi sorting, but when combined with either deletion resulted in a complete loss of this function. The mutant receptors retained the ability to recycle to the Golgi and bind cathepsin D. These results indicate that the cytoplasmic tail of the Man-6-P/IGF-II receptor contains two signals that contribute to Golgi sorting, presumably by interacting with the Golgi clathrin-coated pit adaptor proteins. The Leu-Leu-containing sequence represents a novel motif for mediating interaction with Golgi adaptor proteins.

**I**N many cell types the sorting of newly synthesized acid hydrolases to lysosomes is mediated by the phosphomannosyl recognition system (18, 19). A key step in this process is the binding of the acid hydrolases to mannose 6-phosphate receptors (MPRs)<sup>1</sup> in the Golgi. The receptor-ligand complexes concentrate in clathrin-coated pits and exit from the Golgi in coated vesicles which fuse with an acidified endosomal (prelysosomal) compartment. The low pH of this compartment induces dissociation of the acid hydrolases, which are subsequently packaged into lysosomes. The MPRs either recycle to the Golgi to mediate another round of sorting or move to the cell surface where they may internalize exogenous acid hydrolases via plasma membrane clathrin-coated pits.

Two distinct MPRs have been characterized and shown to participate in the sorting of acid hydrolases in the Golgi. The mannose 6-phosphate/insulin-like growth factor-II receptor (Man-6-P/IGF-II receptor) is a 275-kD type I integral mem-

brane protein that binds both Man-6-P-containing ligands and IGF-II. The other receptor, termed the cation-dependent mannose 6-phosphate receptor (CD-MPR) is also a type I integral membrane glycoprotein with a subunit  $M_r$  of 46,000. The Man-6-P/IGF-II receptor is more efficient than the CD-MPR in sorting acid hydrolases and it accounts for all the internalization of exogenous hydrolases (23, 29, 33).

The signal for the rapid internalization of the Man-6-P/IGF-II receptor at the plasma membrane clathrin-coated pits has been localized to the Y<sup>24</sup>KY<sup>26</sup>SKV<sup>29</sup> sequence in the 163-residue cytoplasmic tail (1, 13, 21). However, the regions of the cytoplasmic tail that allow the receptor to be concentrated in the Golgi clathrin-coated pits are less well understood. Glickman et al. (9) have demonstrated that an immobilized fusion protein containing the cytoplasmic portion of the Man-6-P/IGF-II receptor bound both Golgi and plasma membrane adaptor complexes, which are the components of the clathrin-coated pits that are believed to interact with the cytoplasmic tails of recycling receptors (17, 27, 28). The binding of the plasma membrane adaptors, but not the Golgi adaptors, was dependent on the presence of tyrosines 24 and 26, which serve as critical elements of the internalization signal (9). These data indicate that the Golgi-associated adaptor proteins can interact with additional determinants on the cytoplasmic tail of the receptor. Lobel et al. (21) reported that deletion of the outer 40 amino acids of the cytoplasmic tail of the Man-6-P/IGF-II receptor partially impaired its

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1. *Abbreviations used in this paper:* CD-MPR, cation-dependent mannose 6-phosphate receptor; Man-6-P, mannose 6-phosphate; Man-6-P/IGF-II receptor, mannose 6-phosphate/insulin-like growth factor-II receptor; MEM- $\alpha$ ,  $\alpha$ -minimal essential medium; MPR, mannose 6-phosphate receptor.

sorting function without altering its ability to be rapidly internalized at the plasma membrane. This finding is consistent with the outer portion of the cytoplasmic tail of the receptor containing a signal that is required for efficient interaction with the Golgi adaptors. The fact that the truncated receptor was still able to sort acid hydrolases to some extent raised the possibility that the cytoplasmic tail contains several signals that are required for efficient sorting, and that removal of one of these leads to a partial impairment of this function (21). However, an alternative explanation was that the outer portion of the cytoplasmic tail was required for the return of the receptor to the Golgi, a prerequisite for efficient sorting.

These experiments were designed to further delineate the sorting signals on the cytoplasmic tail of the Man-6-P/IGF-II receptor. Our results indicate that the cytoplasmic tail contains two signals for this function. One involves the carboxyl-terminal four amino acids of the cytoplasmic tail and the other includes the tyrosine-containing internalization signal. By performing the acid hydrolase sorting assays in the presence and absence of Man-6-P in the extracellular medium, we have been able to determine that the block in sorting occurs at the site of interaction with the Golgi clathrin-coated pits.

A preliminary report of these findings has been published (Johnson, K. F., and S. Kornfeld. 1991. *J. Cell Biol.* 115: 244a [Abstr.]).

## Materials and Methods

### Materials

Enzymes used in molecular cloning were obtained from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), or Promega Corp. (Madison, WI). Affinity-purified rabbit anti-bovine Man-6-P/IGF-II receptor antiserum was isolated as previously described (26) and iodinated using the Iodogen procedure as recommended by the manufacturer (Pierce Chemical Co., Rockford, IL). Man-6-P and rabbit anti-human cathepsin D antiserum were provided by Walter Gregory of this laboratory. The expression vector pSFFV-neo was a generous gift of Steven Fine of Washington University, St. Louis, MO (5).  $^{125}\text{I}$ -Na (100 mCi/ml) was obtained from Amersham Corp. (Arlington Heights, IL). EXPRE $^{35}\text{S}^{35}\text{S}$  protein labeling mixture (10 mCi/ml) was obtained from DuPont-New England Nuclear (Boston, MA). All other chemicals were of the highest quality commercially available. Oligonucleotides were synthesized with a solid phase synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA) by the Protein Chemistry Facility of Washington University, St. Louis, MO.

### Mutagenesis of Man-6-P/IGF-II Receptor cDNA

To generate construct 500 (Leu $^{160}$ →STOP $^{160}$ ), a restriction fragment encoding the cytoplasmic tail of the receptor was subcloned into the vector pGBT518 (Gold Biotechnology, Inc., St. Louis, MO) and a single-stranded template was generated from *Escherichia coli* strain CJ236 infected with the helper phage M13K07. This single-stranded template was annealed with an oligonucleotide primer that introduced two sequential stop codons into the receptor cDNA. After primer extension, the plasmids were used to transform *E. coli* strain JM109. Colonies were selected using the AlterGene method (Gold Biotechnology, Inc.). The sequence of the cDNA that had been through a single-stranded intermediate was confirmed by dideoxy sequencing using [ $^{35}\text{S}$ ]dATP and T7 DNA polymerase as recommended by the manufacturer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). A restriction fragment containing the mutated region was subcloned into the expression vector pSFFV-neo-Man-6-P/IGF-II to generate the full-length receptor (1). Construct 501 (Tyr $^{24,26}$ →Ala $^{24,26}$ /Leu $^{160}$ →STOP $^{160}$ ) was made by subcloning the appropriate restriction fragment from construct 500 into construct Ss $_1$  (Tyr $^{24,26}$ →Ala $^{24,26}$ ) (21). The generation of all other con-

structs used in this study and shown in Tables I and II has been described elsewhere (1, 13, 21).

### Transfection of Man-6-P/IGF-II Receptor-deficient Cells

A Man-6-P/IGF-II receptor-deficient mouse L cell line, designated L(Rec $^-$ ), was maintained in  $\alpha$ -minimal essential medium (MEM- $\alpha$ ) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100  $\mu\text{g}/\text{ml}$  streptomycin at 37°C in a humidified 5% CO $_2$  environment (1, 7). The cells were transfected with 20  $\mu\text{g}$  of XbaI-linearized mutant pSFFV-neo-Man-6-P/IGF-II receptor cDNA and selected for resistance to neomycin sulfate (G418) as previously described (21). For each construct 20–30 individual G418-resistant colonies were isolated 10–14 d after transfection and screened for receptor expression by their ability to bind  $^{125}\text{I}$ -labeled, affinity-purified rabbit anti-bovine Man-6-P/IGF-II receptor antiserum as previously described (1, 16). The clones expressing the highest levels of receptor were expanded and maintained in the medium described above supplemented with 350  $\mu\text{g}/\text{ml}$  G418. Typically, two to four colonies were chosen for further study. The level of receptor expression was roughly comparable in most of the cell lines and ranged from 0.18- to 1.7-fold the receptor level in the Cc $_2$  cell line, which expresses the bovine wild-type receptor (Tables I and II).

### Determination of Relative Receptor Levels in Transfected L(Rec $^-$ ) Cells

Confluent cell monolayers in 22-mm wells of a 12-well tissue culture plate were placed in an ice-water bath, washed twice with MEM- $\alpha$ , and then incubated for 3 h on ice in MEM- $\alpha$  containing 25 mM Hepes, pH 7.4, and a saturating amount of affinity-purified anti-Man-6-P/IGF-II receptor antiserum (130 ng of  $^{125}\text{I}$ -labeled [ $8 \times 10^6$  cpm/ $\mu\text{g}$ ] and 330 ng of unlabeled antibody) in the presence or absence of 0.1% saponin (wt/vol). The cells were then washed five times with ice-cold Dulbecco's PBS containing 10 mg/ml BSA (PBS/BSA) and three times with ice-cold PBS, and then solubilized with 0.1 N NaOH. Cell-associated radioactivity was determined on a  $\gamma$ -counter (model 300; Beckman Instruments, Inc., Fullerton, CA). The protein content was determined by the method of Lowry et al. (22) using BSA as the standard. The Man-6-P/IGF-II receptor levels were calculated as counts per minute of antibody bound per microgram cell protein. The levels of mutant receptor are expressed relative to the reference cell line Cc $_2$ , which is transfected with the wild-type bovine Man-6-P/IGF-II receptor cDNA and is arbitrarily assigned a value of 1.0.

### Metabolic Labeling and Sorting of Cathepsin D

Confluent cell monolayers in 24-well tissue culture dishes were rinsed twice with Dulbecco's PBS. The cells were incubated for 30 min at 37°C in 0.2 ml MEM- $\alpha$  lacking methionine and glutamine, 10% dialyzed and heat-inactivated fetal bovine serum, 20 mM Hepes, pH 7.4, 1 mCi/ml EXPRE $^{35}\text{S}^{35}\text{S}$  protein labeling mixture, and 10 mM Man-6-P where indicated. The labeling medium was then removed and 0.4 ml of fresh MEM- $\alpha$ , 10% fetal bovine serum, and 20 mM Hepes, pH 7.4, plus or minus 10 mM Man-6-P was added to each well. After 4 h the cells were placed on ice and the corresponding labeling and chase media samples were pooled. The cells were processed as described (21). The amount of cathepsin D sorted was determined by immunoprecipitating one-half of the cell and media high-speed supernatants with rabbit anti-human cathepsin D antiserum as previously described (4). The immunoprecipitates were analyzed by 10% SDS-PAGE under nonreducing conditions. The gels were soaked in EN $^3$ HANCE (DuPont-New England Nuclear) according to the manufacturer's instructions, dried, and exposed to Kodak X-Omat AR film at  $-70^\circ\text{C}$ . The processed and secreted forms of cathepsin D were excised from the gel using the autoradiograph as a template. The gel slices were prepared as described (24) and the associated radioactivity was determined by liquid scintillation counting on a counter (model 6800; Beckman Instruments, Inc.). The percentage of cathepsin D sorted is equal to the amount of radioactivity in the cell-associated processed form divided by the sum of the radioactivity in the secreted and processed forms.

### Purification and Iodination of $\beta$ -Glucuronidase

The cell line 13.2.1, which overexpresses and secretes human  $\beta$ -glucuronidase, was obtained from Dr. W. Sly, St. Louis University. The  $\beta$ -gluc-

uronidase was purified on a Man-6-P/IGF-II receptor affinity column as described elsewhere (13).

30  $\mu\text{g}$   $\beta$ -glucuronidase was iodinated with 1 mCi of  $^{125}\text{I}$ -Na using soluble lactoperoxidase as described (30). The iodination reaction was applied to a  $1.0 \times 110$  cm Sephadex G-150 column that was previously equilibrated with 0.05 M  $\text{NaPO}_4$ , pH 7.6, 0.15 M NaCl, and 1 mg/ml BSA. The material in the first peak ( $V_e/V_t = 0.40$ ) was pooled, avoiding the leading edge of aggregated material. Assuming complete recovery of the  $\beta$ -glucuronidase, the specific activity of the enzyme is 8–16  $\mu\text{Ci}/\mu\text{g}$ .

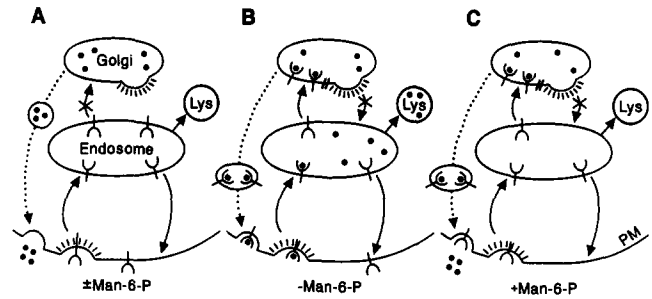
### Release of Prebound Surface-associated $^{125}\text{I}$ - $\beta$ -Glucuronidase

Cells were seeded into six-well tissue culture dishes and assayed when confluent (typically 48–72 h). The cells were incubated for 5 min at  $37^\circ\text{C}$  in MEM- $\alpha$ /10% FBS containing  $2 \times 10^6$  cpm of  $^{125}\text{I}$ - $\beta$ -glucuronidase/ml. The labeling medium was removed and the cells were rapidly rinsed six times with 1 ml of PBS/BSA at  $37^\circ\text{C}$ . The cells in one of the wells were solubilized with 0.1 N NaOH and counted in a  $\gamma$ -counter to determine the total cell-associated radioactivity at zero time. 4 ml of MEM- $\alpha$ /10% FBS was added to each of the other wells, and 0.2-ml aliquots of medium were removed at the times indicated over the next 2 h. The medium samples were centrifuged and the supernatants counted in a  $\gamma$ -counter to determine total released radioactivity and then adjusted to 10% TCA and centrifuged to determine the content of TCA-soluble degraded ligand. This value was subtracted from the total radioactivity to give the value for the released intact  $^{125}\text{I}$ - $\beta$ -glucuronidase. Values obtained with L(Rec<sup>-</sup>) cells served as a measure of nonspecific binding and release and were subtracted from the experimental points. These represented <2% of the specific binding. The percentage of  $\beta$ -glucuronidase released at each time point was determined by dividing the corrected value of released, intact enzyme by the total cell-associated radioactivity at zero time.

## Results

### Use of Man-6-P to Define Sites of Lysosomal Enzyme Sorting Defects

Lobel et al. (21) reported that deleting the outer 40 amino acids of the cytoplasmic tail of the Man-6-P/IGF-II receptor impaired its ability to target newly synthesized cathepsin D to lysosomes, but had no effect on the receptor's rate of internalization at the plasma membrane. This defect in sorting could arise due to a block at either of two different points in the recycling pathway of the receptor. As outlined in Fig. 1, the truncated receptor could be impaired in its movement from an endosomal compartment to the Golgi (Fig. 1 A) or, upon arriving in the Golgi, might be unable to enter the Golgi-derived clathrin-coated pits (Fig. 1, B and C). In the latter instance the receptor would be expected to bind the newly synthesized lysosomal enzymes in the Golgi and then be diverted to the plasma membrane via the constitutive secretory pathway. In the absence of Man-6-P in the extracellular medium, the receptor-ligand complex would be internalized and the lysosomal enzyme targeted to the lysosome via the endocytic pathway (Fig. 1 B). However, if extracellular Man-6-P was present, the ligand would be expected to be displaced at the cell surface, resulting in secretion of the newly synthesized lysosomal enzymes (Fig. 1 C). Alternatively, if the mutant receptor fails to return to the Golgi, the lysosomal enzymes would be secreted, regardless of the presence or absence of Man-6-P in the extracellular medium (Fig. 1 A). Since the secreted enzymes would be diluted into a large extracellular volume, only a small portion would be internalized via surface receptors in the absence of extracellular Man-6-P. The predicted differences in the effects of



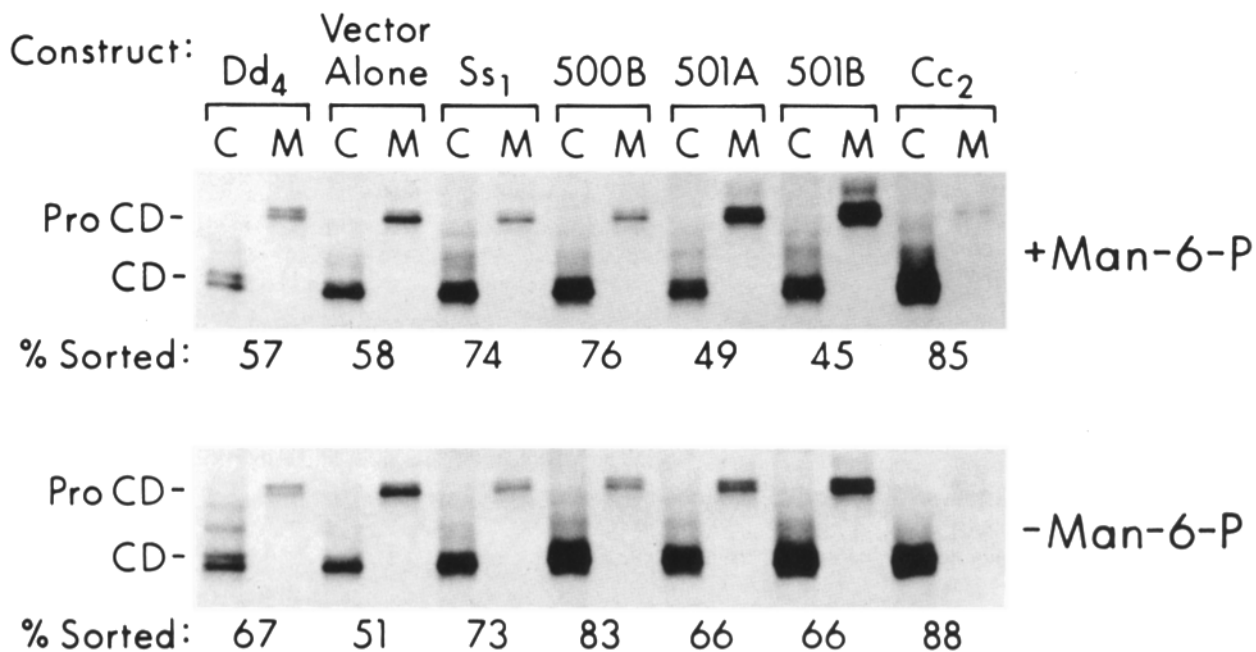
**Figure 1.** Use of Man-6-P to define sites of lysosomal enzyme sorting defects. The inclusion of Man-6-P should have no effect on the sorting of lysosomal enzymes by mutant Man-6-P/IGF-II receptors that are unable to return to the Golgi (A). Mutant Man-6-P/IGF-II receptors that are unable to enter the Golgi clathrin-coated pits should sort lysosomal enzymes in the absence (B) but not in the presence (C) of extracellular Man-6-P.

Man-6-P on lysosomal enzyme sorting can be used to distinguish between these two potential sites of blockage.

### Sorting of Newly Synthesized Cathepsin D in the Presence and Absence of Man-6-P

We initially determined the effect of extracellular Man-6-P on the efficiency of lysosomal enzyme sorting by L(Rec<sup>-</sup>) cells transfected with vector DNA alone (cell line L1) and by L cells expressing the wild-type bovine Man-6-P/IGF-II receptor (cell line Cc<sub>2</sub>). The cells were incubated with [ $^{35}\text{S}$ ]methionine for 30 min and chased for 4 h to allow the newly synthesized cathepsin D to be phosphorylated and either targeted to lysosomes or secreted. Equivalent aliquots of cell homogenates and media were immunoprecipitated and analyzed by SDS-PAGE in order to assess the efficiency of the sorting of cathepsin D. A typical result for these two cell lines is shown in Fig. 2. The cell-associated form of cathepsin D migrates more rapidly than the secreted form due to proteolytic processing of the pro-form to the mature species. This processing begins in the prelysosomal compartment and is completed upon the arrival of the cathepsin D in the lysosome (8). In this experiment, cells transfected with the vector alone sorted 58% of the newly synthesized cathepsin D to lysosomes in the presence of extracellular Man-6-P (10 mM) and 51% in the absence of added Man-6-P. The Cc<sub>2</sub> cells expressing the wild-type bovine Man-6-P/IGF-II receptor sorted 85% of the cathepsin D in the presence of Man-6-P and 88% in its absence. Table I summarizes the results of 42 determinations with these cell lines. The basal level of sorting in the L1 cell line transfected with the vector DNA alone was  $51 \pm 1$  and  $48 \pm 1\%$  in the presence and absence of 10 mM Man-6-P, respectively. The Cc<sub>2</sub> cell line sorted  $84 \pm 1\%$  of the cathepsin D in the presence and  $88 \pm 1\%$  of this enzyme in the absence of extracellular Man-6-P. This difference in sorting in the presence and absence of Man-6-P will be referred to as the sorting differential (Table I). A small sorting differential reflects efficient intracellular targeting of the receptor-ligand complex, while a large difference indicates targeting via the plasma membrane.

We considered the possibility that the absence of Man-6-P from the extracellular medium may allow some portion of



**Figure 2.** Sorting of cathepsin D in the presence and absence of Man-6-P by L cells expressing mutant Man-6-P/IGF-II receptors. Cells were incubated with [<sup>35</sup>S]methionine for 30 min and chased for 4 h. Equivalent samples of a cell detergent extract (C) and the medium (M) were immunoprecipitated with antiserum against cathepsin D and analyzed by 10% SDS-PAGE and fluorography (see Materials and Methods). The positions of the unprocessed procathepsin D (Pro CD) and the proteolytically processed mature cathepsin D (CD) are indicated. The percentage of cathepsin D sorted is listed below each construct. The sorting assays were performed in the presence (*top*) and absence (*bottom*) of 10 mM Man-6-P. The cell lines used are: Dd<sub>4</sub>, L(Rec<sup>-</sup>) cells transfected with Val<sup>8</sup>→STOP<sup>8</sup> Man-6-P/IGF-II receptor cDNA; vector alone, L(Rec<sup>-</sup>) cells transfected with pSFFV-neo DNA; Ss<sub>1</sub>, L(Rec<sup>-</sup>) cells transfected with Tyr<sup>24,26</sup>→Ala<sup>24,26</sup> Man-6-P/IGF-II receptor cDNA; 500B, L(Rec<sup>-</sup>) cells transfected with Leu<sup>160</sup>→STOP<sup>160</sup> Man-6-P/IGF-II receptor cDNA; 501A and 501B, two individual L(Rec<sup>-</sup>) clones transfected with Tyr<sup>24,26</sup>→Ala<sup>24,26</sup>/Leu<sup>160</sup>→STOP<sup>160</sup> Man-6-P/IGF-II receptor cDNA; and Cc<sub>2</sub>, L(Rec<sup>-</sup>) cells transfected with wild-type bovine Man-6-P/IGF-II receptor cDNA.

the secreted cathepsin D to bind to surface receptors and be targeted to lysosomes via the endocytic pathway. To address this issue, Cc<sub>2</sub> cells were incubated with undiluted media containing secretions collected from [<sup>35</sup>S]methionine-labeled

L(Rec<sup>-</sup>) cells. After 4 h at 37°C, the cells were lysed and the amount of cathepsin D taken up from the medium was determined by immunoprecipitation and SDS-PAGE. This experiment showed that only 2% of the added cathepsin D

**Table I.** Sorting of Cathepsin D by L Cells Expressing Mutant Man-6-P/IGF-II Receptors

Mutation	Cell line	Receptor expression*	N‡	Cathepsin D sorted§		Sorting differential
				+ Man-6-P	- Man-6-P	
Vector alone	L1	—	42	51 ± 1	48 ± 1	-3
Wild-type						
...EDLLHV <sup>163</sup>	Cc <sub>2</sub>	1.0	42	84 ± 1	88 ± 1	4
Leu <sup>160</sup> →STOP <sup>160</sup>	500A	1.3	6	75 ± 2	92 ± 1	17
	500B	1.7	5	76 ± 1	90 ± 2	14
	500C	1.1	4	67 ± 2	89 ± 1	22
				Av. = 74 ± 1	Av. = 91 ± 1	Av. = 17
Tyr <sup>24</sup> →Ala <sup>24</sup> / Asn <sup>30</sup> →STOP <sup>30</sup>	344A	1.1	15	75 ± 1	89 ± 1	14
Val <sup>8</sup> →STOP <sup>8</sup>	Dd <sub>4</sub>	1.1	4	44 ± 5	57 ± 4	13
Tyr <sup>24,26</sup> →Ala <sup>24,26</sup>	Ss <sub>1</sub>	0.18	8	70 ± 2	75 ± 1	5
Tyr <sup>24,26</sup> →Ala <sup>24,26</sup> / Leu <sup>159</sup> →STOP <sup>160</sup>	501A	0.54	3	47 ± 4	69 ± 4	22
	501B	0.37	3	43 ± 1	65 ± 4	22
	501C	0.26	1	52	64	12
				Av. = 46 ± 2	Av. = 67 ± 2	Av. = 21

\* Levels of Man-6-P/IGF-II receptor expression in the individual transfected cell lines are expressed relative to the Cc<sub>2</sub> cell line. The Cc<sub>2</sub> cell line contains 0.63 ng receptor/μg membrane protein (21).

‡ N is the number of experiments performed in the sorting assays.

§ Values are expressed as means ± SEM.

|| The sorting differential is determined by subtracting the amount of cathepsin D sorted in the presence of 10 mM Man-6-P from the amount of cathepsin D sorted in its absence.

was internalized. Nevertheless, to minimize the recapture of secreted cathepsin D, the following labeling protocol was used: After the initial 30-min labeling period, the medium (0.2 ml) was removed and replaced with 0.4 ml of chase medium to dilute out any cathepsin D that is secreted. At the end of the experiment the two medium samples were pooled and then immunoprecipitated with anti-cathepsin D antibodies so that the total secretion of cathepsin D would be determined. All values shown in Tables I and II were obtained under these experimental conditions.

### ***Deletion of the Carboxyl-terminal Four Amino Acids of the Cytoplasmic Tail of the Man-6-P/IGF-II Receptor Impairs the Cathepsin D Sorting Function***

In preliminary experiments we found that cells expressing mutant Man-6-P/IGF-II receptors with deletions of the carboxyl-terminal 89, 40, and 12 amino acids of the cytoplasmic tail were partially impaired in sorting cathepsin D when 10 mM Man-6-P was included in the extracellular medium. However, these three mutant receptors sorted cathepsin D as well as the wild-type receptor in the absence of Man-6-P, consistent with a defect in entry into Golgi-coated pits. To more precisely define the residues required for efficient intracellular sorting, we generated a cDNA that encodes a mutant receptor lacking the outer four residues of the cytoplasmic tail (construct 500; Leu<sup>160</sup>→STOP<sup>160</sup>). As shown in Fig. 2, a cell line (500B) expressing this mutant receptor sorted 76% of the cathepsin D in the presence of Man-6-P and 83% in its absence. Table I summarizes the results of 15 sorting experiments with three cell clones (500A, B, and C) that have varying levels of expression of this truncated receptor. The average sorting of cathepsin D was 74 ± 1% in the presence of extracellular Man-6-P and 91 ± 1% in its absence. The large sorting differential (17) indicates that the terminal four amino acids of the cytoplasmic tail are required for efficient intracellular lysosomal enzyme targeting.

### ***Identification of a Second Sorting Signal in the Cytoplasmic Tail of the Man-6-P/IGF-II Receptor***

The finding that deletion of the outer four residues of the cytoplasmic tail only partially abolished the ability of the receptor to mediate intracellular sorting suggested that additional sequences contributed to sorting in the Golgi. Consequently, two additional deletion mutants were analyzed to localize this signal. Cell line 344A expresses a mutant receptor (Tyr<sup>24</sup>→Ala<sup>24</sup>/Asn<sup>30</sup>→STOP<sup>30</sup>) which deletes the outer 134 amino acids of the cytoplasmic tail and exhibits the same behavior as the Leu<sup>160</sup>→STOP<sup>160</sup> construct in the sorting assay.<sup>2</sup> As shown in Fig. 3 and summarized in Table I, this mutant receptor had a sorting differential of 14 (75 ± 1% cathepsin D sorted in the presence of Man-6-P and 89 ± 1% sorted in the absence of Man-6-P). In contrast to these results, construct Dd<sub>4</sub>, which contains only seven amino acids of the cytoplasmic tail (Val<sup>8</sup>→STOP<sup>8</sup>), sorted cathepsin D at the basal level in the presence of Man-6-P (44 ± 5%; Fig. 2 and Table I). In the absence of Man-6-P, the cathepsin D sorting increased to 57 ± 4%. One reason why

2. The substitution of Tyr<sup>24</sup> with an Ala residue has a minimal effect on the rate of endocytosis since Tyr<sup>26</sup> is the critical Tyr in the internalization signal Y<sup>24</sup>KY<sup>26</sup>SKV<sup>29</sup> (1, 13).

this receptor does not sort better in the absence of Man-6-P is that it releases a portion of its bound ligand at the cell surface (see below). These results indicated that the second sorting signal was located between residues 8 and 29 of the cytoplasmic tail.

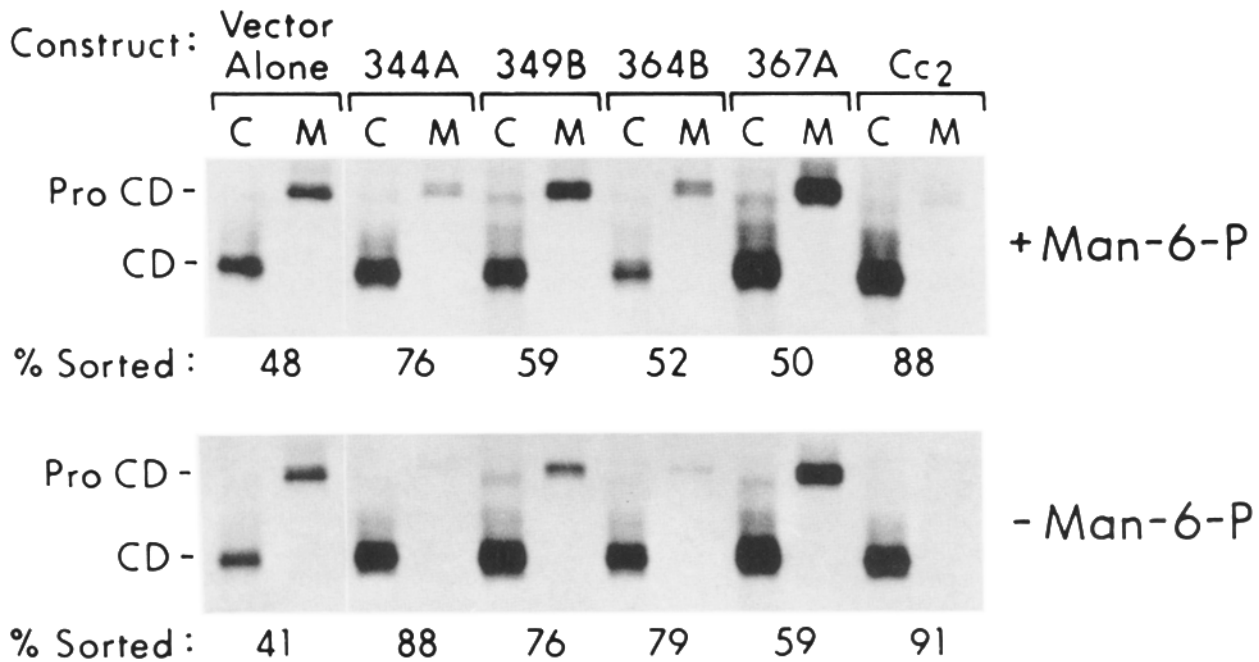
Since the signal for rapid internalization of the receptor had been previously localized to residues 24–29 of the cytoplasmic tail, we postulated that these residues might also serve as the second Golgi sorting signal. A cell line (Ss<sub>1</sub>) expressing a mutant receptor with both tyrosines substituted with alanines (Tyr<sup>24,26</sup>→Ala<sup>24,26</sup>) exhibited a sorting differential similar to that observed with Cc<sub>2</sub>, although the maximal level of sorting was only 75%, presumably reflecting the low expression of this mutant receptor (Fig. 2 and Table I). However, when the outer four residues of the cytoplasmic tail were deleted from a receptor with the Tyr<sup>24,26</sup>→Ala<sup>24,26</sup> mutation (construct 501), the ability to sort cathepsin D in the presence of Man-6-P was completely lost (Fig. 2 and Table I). Furthermore, the sorting of cathepsin D by this mutant receptor increased from 46 ± 2 to 67 ± 2% when Man-6-P was left out of the assays. This result is consistent with Tyr<sup>24</sup> and/or Tyr<sup>26</sup> being a component of the second Golgi sorting signal.

### ***Further Characterization of the Tyrosine-containing Signal***

The fact that construct 344 (Tyr<sup>24</sup>→Ala<sup>24</sup>/Asn<sup>30</sup>→STOP<sup>30</sup>) retains a partial Golgi sorting function implicates Tyr<sup>26</sup> as being a component of the Golgi signal. Further support for this conclusion comes from the result with construct 367A, which is identical to construct 344 except that tyrosine 26 is mutated to an asparagine. As shown in Fig. 3 and summarized in Table II, this mutant receptor sorted cathepsin D at the basal level (50 ± 2%) in the presence of Man-6-P and the sorting improved to 59 ± 3% in the absence of Man-6-P. Substitution of Tyr<sup>24</sup> and Tyr<sup>26</sup> with phenylalanines in the truncated receptor (construct 364B, Tyr<sup>24,26</sup>→Phe<sup>24,26</sup>/Asn<sup>30</sup>→STOP<sup>30</sup>) also prevented cathepsin D sorting in the presence, but not in the absence, of Man-6-P (sorting differential of 26; Fig. 3 and Table II). Cell line 349B expresses a mutant receptor in which the spacing of the YSKV signal has been altered by the insertion of three alanine residues between serine 27 and the lysine initially present at position 28. This cell line sorts 58 ± 2% of cathepsin D in the presence of Man-6-P and 75 ± 2% in the absence of Man-6-P (Fig. 3 and Table II).

### ***Receptors with Defective Internalization Signals Lose Bound Ligand at the Cell Surface***

If the mutant receptors are defective only in entry into Golgi clathrin-coated pits, one would expect them to sort cathepsin D as well as the wild-type receptor when receptor levels are comparable and Man-6-P is not present in the medium (Fig. 1 B). While this did occur with several of the mutant receptors, such as 344 and 500, the sorting was significantly less with other mutant receptors, particularly Dd<sub>4</sub> and 367. These latter receptors exhibit markedly reduced rates of internalization at the plasma membrane (1, 13, 21). This raised the possibility that the receptors might be losing a portion of their bound ligand at the cell surface even in the absence



**Figure 3.** Sorting of cathepsin D in the presence and absence of Man-6-P by L cells expressing truncated Man-6-P/IGF-II receptors. Cells were processed as described in the legend to Fig. 2. The cell lines used are: vector alone, L(Rec<sup>-</sup>) cells transfected with pSFFV-neo DNA; 344A, L(Rec<sup>-</sup>) cells transfected with Tyr<sup>24</sup>→Ala<sup>24</sup>/Asn<sup>30</sup>→STOP<sup>30</sup> (...AKYSKV<sup>29</sup>) Man-6-P/IGF-II receptor cDNA; 349B, L(Rec<sup>-</sup>) cells transfected with ...AKYSAAAKV<sup>32</sup> Man-6-P/IGF-II receptor cDNA; 364B, L(Rec<sup>-</sup>) cells transfected with ...FKFSKV<sup>29</sup> Man-6-P/IGF-II receptor cDNA; 367A, L(Rec<sup>-</sup>) cells transfected with ...AKNSKV<sup>29</sup> Man-6-P/IGF-II receptor cDNA; and Cc<sub>2</sub>, L(Rec<sup>-</sup>) cells transfected with wild-type bovine Man-6-P/IGF-II receptor cDNA.

of extracellular Man-6-P. To determine if this was occurring, the cell lines Cc<sub>2</sub>, Dd<sub>4</sub>, 367, and 501B were incubated with <sup>125</sup>I-β-glucuronidase for 5 min at 37°C to allow ligand binding and then washed and resuspended in fresh media for 2 h to follow the fate of the bound ligand. As shown in Fig. 4, 32 and 25% of the <sup>125</sup>I-β-glucuronidase was released from the 367A and Dd<sub>4</sub> cells, respectively, whereas virtually none of the ligand was released from the Cc<sub>2</sub> and 501A cells. The release of ligand into the media inversely correlates with the relative rate of internalization of these four receptors

(Cc<sub>2</sub> = 1; 501 = 0.25; 367A = 0.01; and Dd<sub>4</sub> = 0.01) (13). However, even when this loss of ligand at the cell surface is taken into account, the receptors containing mutations that drastically impair internalization still exhibit levels of cathepsin D sorting in the absence of Man-6-P that are below those observed with the Cc<sub>2</sub> cell line.

**Phosphorylation of the Cytoplasmic Tail Is Not Necessary for Lysosomal Enzyme Sorting**

The cytoplasmic tail of the bovine Man-6-P/IGF-II receptor

**Table II. Characterization of Tyrosine-containing Sorting Signal**

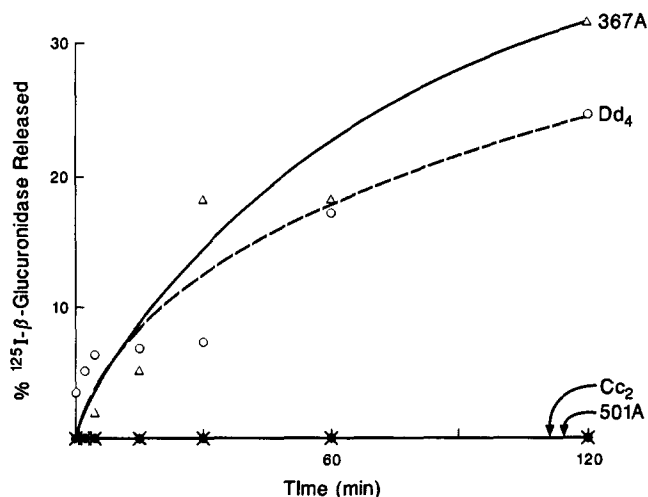
Mutation	Cell line	Receptor expression*	N‡	Cathepsin D sorted§		Sorting differential
				+ Man-6-P	- Man-6-P	
Vector alone	L1	—	42	51 ± 1	48 ± 1	-3
Tyr <sup>24</sup> →Ala <sup>24</sup> / Asn <sup>30</sup> →STOP <sup>30</sup> ...AKYSKV <sup>29</sup>	344A	1.1	15	75 ± 1	89 ± 1	14
...AKNSKV <sup>29</sup>	367A	0.9	4	50 ± 2	59 ± 3	9
...FKFSKV <sup>29</sup>	364B	1.2	4	52 ± 3	78 ± 2	26
...AKYSAAAKV <sup>32</sup>	349B	1.5	4	58 ± 2	75 ± 2	17
S <sup>19</sup> T <sup>40</sup> S <sup>85</sup> S <sup>156</sup> → A <sup>19,40,85,156</sup>	301Y	0.45	5	81 ± 2	85 ± 1	4

\* Levels of Man-6-P/IGF-II receptor expression in the individual transfected cell lines are expressed relative to the Cc<sub>2</sub> cell line. The Cc<sub>2</sub> cell line contains 0.63 ng receptor/μg membrane protein (21).

‡ N is the number of experiments performed in the sorting assays.

§ Values are expressed as means ± SEM.

|| The sorting differential is determined by subtracting the amount of cathepsin D sorted in the presence of 10 mM Man-6-P from the amount of cathepsin D sorted in its absence.



**Figure 4.** Release of surface-bound  $^{125}\text{I}$ - $\beta$ -glucuronidase from L cells expressing mutant Man-6-P/IGF-II receptors. See Materials and Methods for the details of the assay. The total radioactivity bound to each cell line at zero time was: Cc<sub>2</sub> (●) 119,540 cpm; 501B (×) 8,410 cpm; 367A (Δ) 26,540 cpm; Dd<sub>4</sub> (○) 59,890 cpm.

contains four potential phosphorylation sites at Ser<sup>19</sup>, Thr<sup>40</sup>, Ser<sup>85</sup>, and Ser<sup>156</sup>. To determine whether phosphorylation of any of these residues plays a role in the sorting process, we generated a construct in which these four amino acids are replaced with alanine residues. As summarized in Table II, the 301Y cell line which expresses this mutant receptor sorted cathepsin D as well as the wild-type receptor ( $81 \pm 2$  and  $85 \pm 1\%$  cathepsin D sorted in the presence and absence of Man-6-P, respectively). This indicates that phosphorylation of the cytoplasmic tail plays no detectable role in our sorting assay.

## Discussion

In a previous study we demonstrated that mutant Man-6-P/IGF-II receptors lacking 40 or 89 residues from the carboxyl terminus of the cytoplasmic tail functioned normally in endocytosis, but were partially impaired in sorting (21). The sorting assays in that study were performed in the presence of extracellular Man-6-P. We suggested three possibilities to explain the partial loss of the sorting function. One was that the deleted amino acids might be required for efficient return of the receptor from an endosomal compartment to the Golgi. Alternatively, the cytoplasmic tail could contain multiple signals that are required for efficient sorting in the Golgi, and removal of one of these might lead to partial impairment of this function. The third possibility was that the primary sequence required for efficient sorting was still present on the mutant receptors, but was unable to achieve a functional state when the cytoplasmic tail was truncated. The data available at that time did not allow us to distinguish between these possibilities.

By performing the cathepsin D sorting assays in the presence and absence of extracellular Man-6-P, it has been possible to distinguish between these potential defects in receptor function. We found that the direct intracellular targeting of cathepsin D from the Golgi to an acidified endosomal (prelysosomal) compartment (i.e., the route taken by the

wild-type receptor) was unaffected by the addition of Man-6-P to the extracellular medium. However, targeting that occurred via endocytosis of receptor–ligand complexes that had reached the cell surface was sensitive to extracellular Man-6-P. By determining the efficiency of cathepsin D sorting in the presence and absence of Man-6-P (the “sorting differential”), we have been able to evaluate the ability of any given receptor to enter the Golgi clathrin-coated pits. In addition, the efficiency of sorting in the absence of Man-6-P serves as a measure of the return of the receptor to the Golgi since most of the receptors that bind ligand in the Golgi will eventually transport the ligand to lysosomes. The results demonstrate that all of the mutant receptors retain the ability to recycle to the Golgi and bind cathepsin D.

Our data indicate that the cytoplasmic tail of the Man-6-P/IGF-II receptor contains two signals that are required for efficient lysosomal enzyme sorting in the Golgi: the Tyr-containing sequence (residues 24–29) that also functions as an internalization signal and the Leu-Leu-His-Val sequence at the carboxyl terminus of the 163 amino acid cytoplasmic tail. The fact that cathepsin D sorting in the Golgi is only partially impaired when just the Leu-Leu-His-Val<sup>163</sup> sequence is deleted and minimally altered when only tyrosines 24 and 26 are substituted with alanines indicates that each sorting signal is capable of functioning independently rather than one sequence being absolutely necessary for the functioning of the other. It is only when both signals are altered, as in construct 501, that Golgi sorting is completely lost. How might two different regions of the cytoplasmic tail of the Man-6-P/IGF-II receptor play a role in Golgi sorting? The findings are most consistent with there being two independent sites of interaction between the cytoplasmic tail of the Man-6-P/IGF-II receptor and the Golgi adaptor complex of the clathrin-coated pits. This would explain the observation of Glickman et al. (9) that the cytoplasmic tail of the Man-6-P/IGF-II receptor retains the ability to interact with the Golgi adaptor complex even when tyrosines 24 and 26 are mutated to alanines. However, we cannot exclude the alternative explanation that the Leu-Leu-His-Val motif interacts with the Tyr-containing motif to enhance binding of the latter to the Golgi adaptor complex.

While most of the mutant receptors that exhibited impairment in their Golgi sorting function were able to target cathepsin D quite efficiently when the assays were performed in the absence of Man-6-P, there were two exceptions (Dd<sub>4</sub> and 367A). We believe that these mutant receptors illustrate the limitation of the approach, which is the requirement that there be sufficient receptor molecules available to return to the Golgi to bind the bulk of the newly synthesized lysosomal enzymes. Both of these mutant receptors are very defective in internalization at the plasma membrane and as a result they accumulate on the cell surface. One consequence is that the receptor molecules that do acquire ligand in the Golgi and then move to the plasma membrane lose a portion of their bound ligand into the medium (Fig. 4). But this cannot account for the lower than expected sorting differential observed with these receptors. Rather, the most likely explanation is that an insufficient number of receptor molecules enter the intracellular pool, resulting in an inadequate number of molecules returning to the Golgi to bind the newly synthesized lysosomal enzymes. Nevertheless, both of these receptors did exhibit sorting differentials that were greater than

**Table III. Putative Golgi Sorting Signals**

lgp-A (2, 3, 6, 10, 12)	RKRSHAG YQTI
lgp-B (rat/mouse) (10, 12)	KRHHTG YEQF
lgp-B (human) (12)	KHHHAG YEQF
Bovine Man-6-P/IGF-II receptor	NVSYK <sup>26</sup> YSKV.....LLHV <sup>163</sup>
Bovine CD-MPR (15)	...HLLPM <sup>67</sup>
Rat LIMP II (31, 32)	...LIRT <sup>20</sup>
T cell antigen receptor γ chain (20)	DKQTLL <sup>131</sup> ...YQPL <sup>140</sup>

Amino acids shown to be important for Golgi sorting are designated in bold type.

observed with the wild-type receptor, especially when a correction was made for the release of bound ligand into the medium. This was only a problem with mutant receptors that had a very severe impairment in their rate of internalization at the plasma membrane.

The observation that the Tyr<sup>24</sup>-Lys-Tyr<sup>26</sup>-Ser-Lys-Val<sup>29</sup> internalization sequence also functions as a Golgi sorting sequence has similarities to what has been observed in the targeting of lysosomal membrane glycoproteins to lysosomes (19). Most of the members of this family of proteins have short cytoplasmic tails that terminate in either Gly-Tyr-Gln-Thr-Ile or Gly-Tyr-Glu-Gln-Phe sequences (Table III). Harter and Mellman (11) have shown that rat lgp A is normally transported directly from the Golgi to lysosomes, but if the glycine at position 7 of the cytoplasmic tail is mutated to an alanine, the lgp A is diverted from the Golgi to the cell surface and then internalized via plasma membrane clathrin-coated pits. This internalization requires the presence of the tyrosine residue at position 8 (34). Thus, in this protein the Tyr-Gln-Thr-Ile sequence serves as both a plasma membrane internalization signal and a Golgi sorting signal, although it functions in the latter instance only when the glycine is present.

The finding that the carboxyl-terminal Leu-Leu-His-Val residues of the cytoplasmic tail are required for efficient Golgi sorting is of particular interest in view of our recent studies with the CD-MPR. We have observed that deletion of the carboxyl-terminal His-Leu-Leu-Pro-Met residues of the cytoplasmic tail of this receptor abolishes its sorting function without altering its rate of internalization or its ability to return to the Golgi (15; Johnson, K. F., and S. Kornfeld. 1991. *J. Cell Biol.* 115:244a [Abstr.]). Alanine scanning experiments revealed that the His-Leu-Leu residues were the critical elements required for the Golgi sorting function. As shown in Table III, the Leu-Leu sequence is located in the same position in both receptors relative to the carboxyl terminus.

Further evidence that the di-leucine motif functions as a Golgi sorting sequence comes from the studies of Letourneur and Klausner (20) on the T cell antigen receptor γ and δ chains. These investigators identified two distinct sequences of the cytoplasmic tails of these subunits, a di-

leucine motif and a tyrosine-containing motif, which, when transplanted together onto the Tac antigen (IL-2 receptor α chain), caused the chimeric protein to be delivered to lysosomes without going to the cell surface. Chimeras containing only one of the motifs were still delivered to lysosomes, but a portion of the molecules moved from the Golgi to the plasma membrane where they underwent endocytosis. The di-leucine sequence was shown to be the essential element of a six amino acid motif, DKQTLL (Table III). The finding that both sequences are required for efficient sorting of the chimeric protein in the Golgi is similar to our data with the Man-6-P/IGF-II receptor. Letourneur and Klausner (20) also presented evidence that the di-leucine-containing motif functions as an internalization signal at the plasma membrane.

In addition to the two MPRs and the T cell antigen receptor γ and δ chains, a similar motif is present in the cytoplasmic tail of a lysosomal membrane protein called LIMP II, which is also transported from the Golgi to lysosomes by an intracellular route (31, 32). This glycoprotein has a 20 amino acid residue cytoplasmic tail that lacks a tyrosine residue but contains a carboxyl-terminal Leu-Ile-Arg-Thr sequence (Table III). This cytoplasmic tail is sufficient for lysosomal targeting (31). The only obvious similarity between the cytoplasmic tails of the MPRs and LIMP II are the Leu-Leu and Leu-Ile sequences near the carboxyl termini. Taken together, these findings indicate that the Leu-Leu (or Leu-Ile) sequence may serve as a general motif for sorting membrane proteins in the Golgi. Whether the Leu-Leu sequence in the MPRs functions independently or as part of a larger recognition motif cannot be determined from our data.

The cytoplasmic tail of the Man-6-P/IGF-II receptor also contains four potential serine/threonine phosphorylation sites, two of which are casein kinase II sites. Meresse et al. (25) have recently described a casein-kinase II-like activity associated with the 47-kD subunit of the Golgi adaptor complex. This purified kinase activity was able to phosphorylate the two casein-kinase II-like sites on the Man-6-P/IGF-II receptor (Ser<sup>85</sup> and Ser<sup>156</sup>) in vitro. In addition, these two residues were shown to be phosphorylated in vivo in Madin-Darby bovine kidney cells. However, our experiments demonstrated that mutagenesis of all four potential phosphorylation sites had no detectable effect on the sorting of cathepsin D in either the presence or absence of extracellular Man-6-P. While this result excludes an absolute requirement for receptor phosphorylation in the sorting of cathepsin D, the experiment does not preclude a role for phosphorylation in the regulation of receptor function on a shorter time scale or under a different set of experimental conditions.

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