THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 11, Issue of March 14, pp. 7003–7012, 1997

Printed in U.S.A.

Systematic Mutational Analysis of the Cation-independent Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor Cytoplasmic Domain

AN ACIDIC CLUSTER CONTAINING A KEY ASPARTATE IS IMPORTANT FOR FUNCTION IN LYSOSOMAL ENZYME SORTING*

(Received for publication, August 13, 1996, and in revised form, December 27, 1996)

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We have used systematic mutational analysis to identify signals in the 166-residue murine cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain required for efficient sorting of lysosomal enzymes. Alanine cluster mutagenesis on all conserved residues apart from the endocytosis signal demonstrates that the major sorting determinant is a conserved casein kinase II site followed by a dileucine motif (^{157}DDSDEDLL^{164}). Small deletions or additions outside this region have severe to mild effects, indicating that context is important. Single residue mutagenesis indicates that cycles of serine phosphorylation/dephosphorylation are not obligatory for sorting. In addition, the two leucine residues and four of the five negatively charged residues can readily tolerate conservative substitutions. In contrast, aspartate 160 could not tolerate isoelectric or isosteric substitutions, implicating it as a critical component of the sorting signal.

An important question in cell biology is how itinerant proteins are properly routed between different intracellular compartments. One particularly interesting example is the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R),[1] which mediates targeting of lysosomal enzymes by two distinct pathways (reviewed in Refs. 1 and 2). In one pathway, the receptor functions in endocytosis of extracellular ligands. In the other, the receptor functions in the direct intracellular targeting of newly synthesized lysosomal enzymes ("sorting"). There are multiple molecular events that occur in each transport pathway. Like many other endocytic receptors, M6P/IGF2Rs at the cell surface bind ligands, cluster into clathrin-coated transport vesicles, and travel to an acidic prelysosomal compartment. However, unlike most other receptors, in the intracellular targeting pathway, M6P/IGF2Rs in the Golgi apparatus bind newly synthesized mannose 6-phosphorylated lysosomal enzymes, cluster into clathrin-coated transport vesicles, and travel to an acidic prelysosomal compartment, where the endocytic and intracellular targeting pathways converge. Here, the low pH promotes dissociation of the receptor-ligand complex, and the free receptors recycle back to the plasma membrane or Golgi apparatus to function in additional rounds of targeting.

As a step toward understanding the biochemical mechanisms underlying these processes, we are characterizing the determinants on the M6P/IGF2R important for intracellular trafficking. The mature murine receptor has an amino-terminal 2270-residue extracytoplasmic domain, a single 23-residue membrane-spanning region, and a 166-residue carboxyl-terminal cytoplasmic domain. While some targeting information may reside in the extracytoplasmic region (3, 4), the cytoplasmic domain contains determinants required for both endocytosis and sorting. Like many other endocytic receptors, rapid internalization of the M6P/IGF2R requires a tyrosine-based motif. This determinant is located in the first 30 residues of the cytoplasmic domain and has been extensively characterized (5–7). In contrast, the determinants required for sorting are less well understood, but at least in part require residues located in the C-terminal region of the cytoplasmic domain (5, 8, 9).

In a previous study (8), we found that the cytoplasmic sorting signals are conserved among bovine, human, and murine M6P/IGF2Rs, and a consensus casein kinase II site followed by two leucines near the C terminus is important for sorting. However, the cytoplasmic domain contains multiple other conserved sequences that may be important, including a second consensus casein kinase II site followed by valine and leucine. In this study, we have systematically analyzed the function of the conserved residues of the M6P/IGF2R and extensively characterized the key sorting signal.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used in molecular cloning were obtained from New England Biolabs Inc., Life Technologies, Inc., or Stratagene. Geneticin (G418) was obtained from Life Technologies, Inc. Na^{125}I (NEZ-033A; ~17 Ci/mg) was from DuPont NEN. Monoclonal antibodies DM32g and DM86f7, which recognize different epitopes on the extracellular domain of the bovine M6P/IGF2R, were generous gifts from Dr. D. Messner (University of Rochester Medical School). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories and was heat-inactivated prior to use. M6P/IGF2R-depleted FBS was prepared as described previously (10). The mouse L(Rec$^-$) cell line 13.2.1 overproducing human β-glucuronidase was kindly provided by Dr. W. Sly (St. Louis University), and the phosphorylated form was purified as described (6). Antibodies and β-glucuronidase were iodinated using soluble lactoperoxidase as described (11).

Cell Culture—Hepes-buffered DMEM/Ham's F-12 medium (DMEM/

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[1] The abbreviations used are: M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor II receptor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

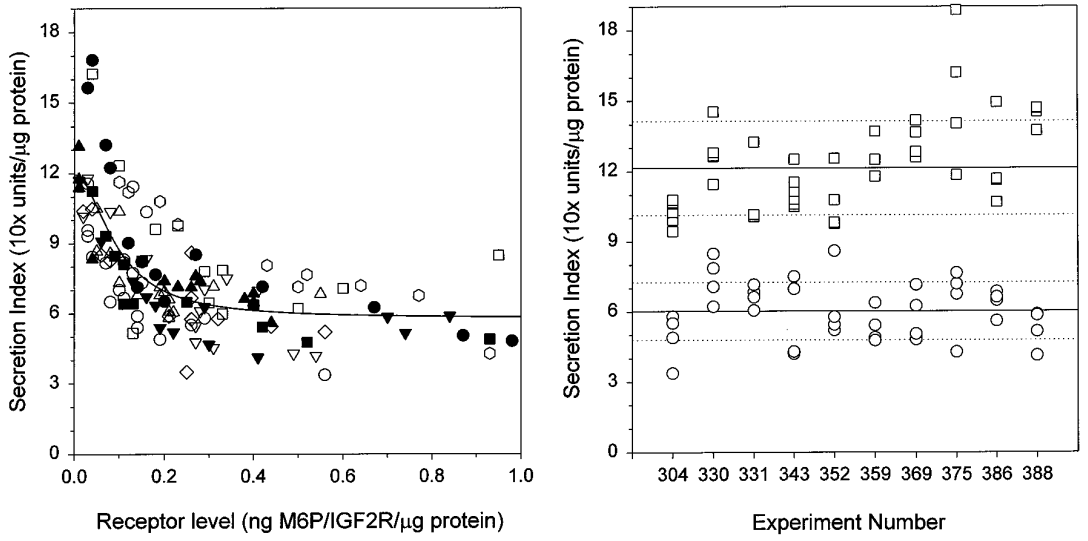

FIG. 1. **Parameters used for classification of mutant receptors.** *Left panel*, positive controls from 10 independent transfection experiments are shown. Each experiment is represented by a different symbol. Curve fitting was performed using a four-parameter logistic regression program (Sigmaplot 3.0, Jandel Scientific). *Right panel*, secretion indices of negative control cell lines (*open squares*) and four positive control cell lines containing the highest levels of receptor (*open circles*) are shown for each experiment. Mean ± S.D. values are indicated by the *solid* and *dotted lines*.

F-12) containing bicarbonate, without phenol red, was made from DMEM/Ham's F-12 base (Sigma D9785) and other cell culture-grade biochemicals (Sigma) according to the supplier's directions. L(CIMPR$^-$)D9, a clonal derivative of a mouse M6P/IGF2R-deficient L cell line (12), has been described previously (8). Cells were routinely grown in DMEM/F-12 and 10% FBS at 37 °C in 5% humidified CO_2. Transfected cells were maintained in the presence of 350 μg/ml (active concentration) G418. When assaying for function of the receptor in lysosomal enzyme targeting, fresh glutamine was added to glutamine-deficient medium to minimize production of ammonia that may have lysosomotropic effects. For surface distribution and internalization assays, frozen stocks were thawed and expanded, and cells were plated at the same cell numbers and grown to confluence prior to assay.

DNA Constructs—The portions of the murine M6P/IGF2R used in truncation mutants Δ4, Δ13, Δ17, and Δ53 were synthesized using the unidirectional nested deletion strategy described previously (8). The other constructs were synthesized by oligonucleotide-directed (13) or polymerase chain reaction-mediated (14) mutagenesis. Sequences of the oligonucleotide primers are compiled in Ref. 15. The template was the previously described fragment of the murine M6P/IGF2R cytoplasmic domain that contained 20 nucleotides of 3′-noncoding sequence (8). Regions that went through single-stranded intermediates or had been amplified by polymerase chain reaction were sequenced to confirm the absence of adventitious changes. A *Bam*HI-*Mlu*I fragment containing the mutated sequence was ligated with a 6943-nucleotide *Eco*RI-partial *Bam*HI fragment of the bovine M6P/IGF2R and a 7.2-kilobase *Eco*RI-*Mlu*I fragment of the pSFFVneo expression vector (16) modified to contain a 3′-*Mlu*I site (8) to generate the final construct. The full-length receptor chimera (referred to as the wild-type receptor) encodes the bovine signal sequence (44 residues) and most of the bovine extracytoplasmic domain (2221 residues) followed by murine sequences (45 residues of extracytoplasmic domain and complete transmembrane and cytoplasmic domains).

Sorting Assay—The details of the sorting assay have been described previously (8). For each experiment, the same passage of L(CIMPR$^-$)D9 cells were thawed and transfected in parallel with the pSV2neo selection marker, wild-type bovine/murine M6P/IGF2R in pSFFVneo, and different receptor mutants in pSFFVneo. Within 2 weeks after selection with G418, for each construct, 32–48 colonies were isolated using cloning cylinders and analyzed for receptor expression and homogeneity by immunofluorescence. Select clones were assayed for secretion of phosphorylated lysosomal enzymes, total cellular protein, and receptor levels. Representative clones were expanded and frozen for later use.

Surface Distribution Assay—Confluent cells in 24-well plates were transferred to an ice-water bath and washed once with ice-cold PBS. One set of duplicate wells were washed twice with DMEM/F-12 and 10% M6P/IGF2R-depleted FBS and then incubated with ~750 ng of monoclonal antibody DM86f (a mixture of ~10^6 cpm iodinated antibody and unlabeled antibody) in 0.3 ml of DMEM/F-12 and 10% M6P/IGF2R-depleted FBS for 90 min. A second set of duplicate wells were treated identically, except the wash and incubation solutions contained 0.3% saponin. Cells were washed four times with PBS and 1% bovine serum albumin and four times with PBS, solubilized with 0.5 ml of 0.1 N NaOH, and counted in a γ-counter. The radioactivity associated with cells treated with and without 0.3% saponin represents total and cell-surface receptor, respectively.

Internalization Assay—Short-term internalization of affinity-purified phosphorylated β-glucuronidase was conducted using a modification of the procedure described (6). Confluent cells in 12-well plates were transferred to an ice-water bath and sequentially washed once with ice-cold PBS; once with DMEM/F-12, 5 mM mannose 6-phosphate, and 10% M6P/IGF2R-depleted FBS; and twice with DMEM/F-12 and 10% M6P/IGF2R-depleted FBS. Cells were incubated with ~2.5×10^6 cpm ^{125}I-labeled β-glucuronidase in 0.5 ml of ice-cold DMEM/F-12 and 10% M6P/IGF2R-depleted FBS for 60 min. The radioactive β-glucuronidase was removed, and cells were washed four times with ice-cold PBS and 1% bovine serum albumin and six times with PBS. To initiate endocytosis, plates were placed in a 37 °C water bath, and 0.5 ml of 37 °C DMEM/F-12 medium was added. At the indicated time points, the medium was collected, and cell-surface β-glucuronidase was stripped using 0.5 ml of mannose 6-phosphate/trypsin solution at pH 5 (6). After 5 min, 0.5 ml of DMEM/F-12 and 10% M6P/IGF2R-depleted FBS was added, and the mixture was transferred to 1.2-ml polypropylene tubes in 96-tube carrier racks. The supernatants were collected after 5 min of centrifugation at 800 × *g*. Cell pellets, stripped supernatants, and media were counted in a γ-counter. Data in Fig. 9 are plotted as cell-associated radioactivity normalized to total counts (pellet + stripped supernatant + medium, averaged over all time points). Internalization rate constants were calculated using the 15-, 30-, 45-, and 60-s time points and are based on the amount of radioactivity internalized over a given time interval, normalized to the average surface counts at the beginning and end of the time interval.

RESULTS

Classification of Mutants

Mutant receptors were analyzed by evaluating their ability to correct hypersecretion of phosphorylated lysosomal enzymes from a mouse L cell line that lacks endogenous M6P/IGF2R as described previously (8). For each mutant, 8–15 stable transfectants were analyzed for receptor levels and secretion of phosphorylated lysosomal enzymes. High concentrations of mannose 6-phosphate were included in the culture medium to prevent indirect targeting through the endocytic pathway. In addition, control experiments were performed to verify that

even severely impaired mutants folded properly as ascertained by their intracellular distribution and ability to bind ligand and to function in endocytosis.

This study is based on analysis of >1000 clonal cell lines generated over the course of 10 independent experiments. In each experiment, up to eight different mutants were transfected and analyzed in parallel along with positive (wild-type receptor) and negative (selection marker alone) controls. Examination of the positive controls indicates that the secretion index (24-h secretion of phosphorylated lysosomal enzymes normalized to total cellular protein) decreases and plateaus with increasing receptor expression (Fig. 1, *left panel*). As an indication of the plateau value, we have selected, from each experiment, the four clones that express the highest levels of receptor. Comparison of the positive controls with all clones expressing selection marker alone indicates that expression of saturating levels of wild-type receptor decreases secretion of lysosomal enzymes by a factor of 2 (Fig. 1, *right panel*).

To help classify the effect of different mutations, we developed a numerical index to compare the relative ability of a given construct and the wild-type receptor to correct secretion of lysosomal enzymes from the receptor-deficient cells. This index, the sorting efficiency (E), is given by the expression $E_x = (S_{neo} - S_x)/(S_{neo} - S_{wt})$, where S_{neo} denotes the average secretion index of all negative control clones, S_x denotes the average secretion index for a given construct of the four clones containing the highest receptor levels, and S_{wt} denotes the grand average secretion of the four positive control clones that contain the highest level of receptor from each of the 10 experiments. Using this index, the sorting efficiency of the normal receptor determined in 10 independent experiments ranges from 0.77 to 1.18 (1.00 ± 0.12, mean ± S.D.) (Table I). Initially, we classified mutants with sorting efficiencies above 0.76 as not significantly impaired (within 2 S.D. of the wild-type mean), those with sorting efficiencies ranging from 0.64 to 0.76 as borderline-impaired (falling between 2 and 3 S.D. below the mean), and those with sorting efficiencies below 0.64 as impaired. As these assignments utilize only a portion of the data and can be influenced by individual clones that exhibit extreme variation, we also inspected the individual scattergrams of secretion index plotted against receptor levels to determine if the mutant appeared significantly different from the corresponding wild-type positive control.

The C-terminal Region Contains a Major Sorting Determinant

To identify determinants on the cytoplasmic domain of the M6P/IGF2R required for efficient intracellular sorting of lysosomal enzymes, we systematically mutated groups of conserved residues to alanines (Fig. 2). Residues important for endocytosis are known to impair sorting (5, 9), but were excluded from this analysis as it is difficult to experimentally distinguish direct from indirect effects, as discussed previously (8). Of the 37 alanine cluster mutations analyzed, 29 had little or no effect on sorting efficiency (Table I), and inspection of the scattergrams was consistent with this conclusion (see below and data not shown). (Scattergrams of all constructs analyzed in this study are compiled in Ref. 15.)

We also classify AC13 as not significantly impaired, despite its having a borderline sorting efficiency. For this mutant, at a given receptor level, most of the clones function similarly to the positive controls transfected and analyzed in parallel (Fig. 3, *top left panel*). The apparently impaired sorting efficiency (E = 0.71 ± 0.72) can be attributed to high secretion from a single clone, which, if dropped from the calculation, would yield E = 1.07 ± 0.07.

TABLE I
Sorting efficiencies of the wild-type receptor and mutants analyzed in this study

Construct	Exp.	Sorting efficiency	Secretion index	Receptor
			10 × units/μg	*ng/μg*
Alanine cluster mutants				
AC01	304	0.45 ± 0.14	9.37 ± 0.83	0.44 ± 0.29
AC02	330	0.03 ± 0.26	11.97 ± 1.60	0.81 ± 0.16
AC02	359	0.51 ± 0.10	9.01 ± 0.60	0.90 ± 0.49
AC03	359	0.54 ± 0.25	8.83 ± 1.55	0.15 ± 0.01
AC03	375	0.22 ± 0.25	10.80 ± 1.50	0.41 ± 0.44
AC04	304	1.19 ± 0.22	4.86 ± 1.37	1.02 ± 0.41
AC05	331	0.90 ± 0.21	6.63 ± 1.31	0.53 ± 0.07
AC06	331	0.82 ± 0.44	7.13 ± 2.72	0.25 ± 0.11
AC07	331	1.19 ± 0.10	4.83 ± 0.61	1.42 ± 0.23
AC08	331	1.13 ± 0.07	5.25 ± 0.44	0.60 ± 0.04
AC09	359	0.81 ± 0.13	7.16 ± 0.82	0.38 ± 0.31
AC10	352	0.97 ± 0.05	6.22 ± 0.28	0.58 ± 0.05
AC11	369	0.95 ± 0.06	6.33 ± 0.36	1.06 ± 0.39
AC12	369	0.80 ± 0.35	7.21 ± 2.13	0.57 ± 0.44
AC13	369	0.71 ± 0.72	7.79 ± 4.41	0.54 ± 0.63
AC14	369	0.93 ± 0.39	6.46 ± 2.41	0.63 ± 0.41
AC15	359	0.98 ± 0.13	6.12 ± 0.80	0.71 ± 0.19
AC16	304	1.23 ± 0.03	4.60 ± 0.19	0.61 ± 0.20
AC17	304	1.18 ± 0.03	4.90 ± 0.21	0.45 ± 0.16
AC18	304	0.89 ± 0.06	6.69 ± 0.39	0.21 ± 0.03
AC19	304	1.08 ± 0.09	5.56 ± 0.55	0.42 ± 0.22
AC20	359	0.65 ± 0.13	8.13 ± 0.79	0.42 ± 0.19
AC21	359	1.02 ± 0.15	5.89 ± 0.93	1.11 ± 0.21
AC22	352	0.93 ± 0.14	6.46 ± 0.87	0.47 ± 0.12
AC23	369	1.03 ± 0.13	5.86 ± 0.78	0.92 ± 0.31
AC24	359	0.89 ± 0.12	6.67 ± 0.73	0.77 ± 0.54
AC25	352	1.04 ± 0.05	5.78 ± 0.29	0.33 ± 0.14
AC26	369	0.97 ± 0.08	6.22 ± 0.46	0.79 ± 0.13
AC27	331	1.01 ± 0.17	5.93 ± 1.06	0.60 ± 0.37
AC28	352	0.73 ± 0.17	7.67 ± 1.03	0.43 ± 0.19
AC29	331	1.04 ± 0.12	5.76 ± 0.74	0.64 ± 0.23
AC30	352	0.98 ± 0.09	6.15 ± 0.55	0.41 ± 0.14
AC31	331	1.18 ± 0.14	4.95 ± 0.84	0.47 ± 0.08
AC32	359	0.98 ± 0.18	6.17 ± 1.13	0.61 ± 0.17
AC33	330	0.54 ± 0.38	8.86 ± 2.35	0.32 ± 0.13
AC34	352	1.14 ± 0.06	5.14 ± 0.34	0.88 ± 0.29
AC35	352	0.89 ± 0.13	6.68 ± 0.82	0.24 ± 0.06
AC36	330	0.79 ± 0.27	7.31 ± 1.65	0.71 ± 0.41
AC37	352	0.69 ± 0.11	7.92 ± 0.67	0.73 ± 0.12
$^{1}KK^{2} \rightarrow AA$	388	1.11 ± 0.09	5.33 ± 0.53	0.72 ± 0.17
$^{4}RR^{5} \rightarrow AA$	388	1.09 ± 0.07	5.45 ± 0.42	0.91 ± 0.16
Deletion and insertion mutants				
Δ13	343	0.25 ± 0.13	10.60 ± 0.79	0.84 ± 0.16
Δ17	343	0.29 ± 0.20	10.36 ± 1.21	0.81 ± 0.14
Δ2	388	0.53 ± 0.20	8.88 ± 1.23	0.81 ± 0.28
Δ4	343	0.39 ± 0.22	9.77 ± 1.32	0.98 ± 0.20
Δ53	343	0.40 ± 0.14	9.69 ± 0.84	0.85 ± 0.05
Δ79(+LLHI)	388	0.44 ± 0.17	9.42 ± 1.05	0.99 ± 0.44
LL(+AA)HI	388	0.86 ± 0.26	6.86 ± 1.58	0.96 ± 0.27
LLHI(+A)	388	0.97 ± 0.10	6.20 ± 0.61	1.09 ± 0.50
LLHI(+AAA)	388	0.61 ± 0.07	8.41 ± 0.40	0.44 ± 0.20
Substitution mutants				
L164I	369	1.03 ± 0.44	5.82 ± 2.72	0.59 ± 0.36
L163I	375	0.88 ± 0.14	6.78 ± 0.89	0.84 ± 0.49
L163V	375	0.72 ± 0.06	7.75 ± 0.36	1.05 ± 0.01
D162N	386	0.73 ± 0.09	7.69 ± 0.53	0.58 ± 0.37
D162E	386	0.96 ± 0.22	6.28 ± 1.36	0.44 ± 0.15
E161D	386	1.23 ± 0.10	4.64 ± 0.61	0.35 ± 0.21
E161Q	386	0.43 ± 0.27	9.51 ± 1.67	0.19 ± 0.02
D160E	386	0.30 ± 0.13	10.30 ± 0.79	0.75 ± 0.20
D160N	386	0.31 ± 0.18	10.26 ± 1.12	0.62 ± 0.13
S159A	386	0.64 ± 0.19	8.25 ± 1.16	0.45 ± 0.09
S159C	375	0.78 ± 0.13	7.39 ± 0.80	0.97 ± 0.23
S159D	375	1.15 ± 0.13	5.08 ± 0.80	1.07 ± 0.21
S159E	375	0.99 ± 0.07	6.07 ± 0.40	1.59 ± 0.24
S159T	375	0.89 ± 0.13	6.67 ± 0.81	1.16 ± 0.25
D158E	386	1.20 ± 0.11	4.81 ± 0.70	0.68 ± 0.31
D158N	375	1.03 ± 0.04	5.86 ± 0.25	1.23 ± 0.25
D157E	386	1.13 ± 0.06	5.25 ± 0.39	0.51 ± 0.14
D157N	375	0.92 ± 0.17	6.52 ± 1.03	1.32 ± 0.30
Wild-type receptor				
WT	304	1.18 ± 0.18	4.89 ± 1.08	0.33 ± 0.16
WT	330	0.77 ± 0.16	7.40 ± 0.98	0.60 ± 0.26
WT	331	0.90 ± 0.08	6.66 ± 0.46	0.32 ± 0.16
WT	343	1.05 ± 0.28	5.73 ± 1.74	0.45 ± 0.09
WT	352	0.96 ± 0.26	6.25 ± 1.58	0.40 ± 0.13
WT	359	1.11 ± 0.12	5.36 ± 0.73	0.57 ± 0.25
WT	369	1.03 ± 0.18	5.81 ± 1.09	0.74 ± 0.25
WT	375	0.93 ± 0.25	6.46 ± 1.51	0.72 ± 0.18
WT	386	0.94 ± 0.09	6.39 ± 0.55	0.41 ± 0.03
WT	388	1.13 ± 0.13	5.25 ± 0.82	0.67 ± 0.18

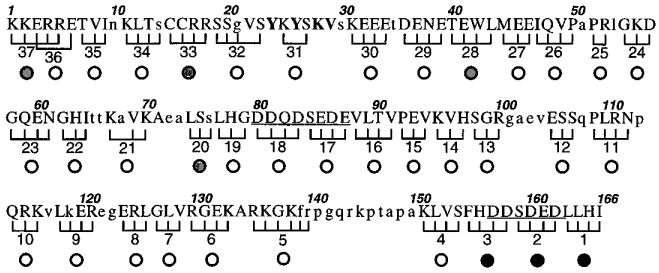

FIG. 2. **Sorting function of the alanine cluster mutants.** Residues of the mouse M6P/IGF2R cytoplasmic domain are *upper-case* if they are identical or conserved in the mouse, cow, and human sequences (8). Residues important for endocytosis are in *boldface*; casein kinase II sites are *underlined*. The identity of each alanine cluster mutant and its function in sorting relative to the wild-type receptor are shown below the sequence. Classification of mutants is described under "Results." *Open circles*, not significantly impaired; *shaded circles*, borderline-impaired; *closed circles*, impaired.

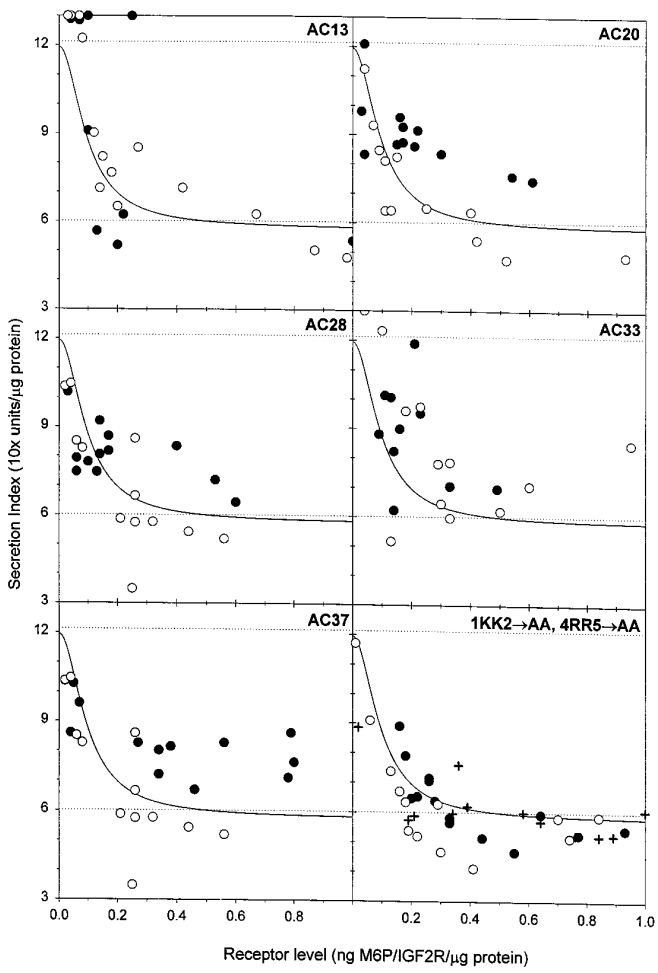

FIG. 3. **Effect of alanine cluster mutations on sorting.** The function of L(CIMPR$^-$)D9 cells expressing a given mutant (*closed circles*) is compared with that of cells expressing the wild-type receptor (*open circles*), with each panel depicting cells that were transfected and analyzed in parallel. The *solid curve* represents the best fit value for all positive controls (see Fig. 1, *left panel*); the *upper* and *lower dotted reference lines* represent the average secretion indices of all negative and select positive controls (see Fig. 1, *right panel*). For clarity of presentation, clones with secretion indices >13 or receptor levels >1 are plotted on the *upper* and *right axes*, respectively. In the *bottom right panel*, *closed circles* and *plus signs* represent the ^{1}KK^2 → AA and ^{4}RR^5 → AA mutations, respectively.

The classification of AC33 as borderline-impaired is tentative. The impaired sorting efficiency index (E = 0.54 ± 0.38) can be partially ascribed to a paucity of clones that express relatively high levels of receptor, and at a given steady-state level of receptor, the majority of the AC33 clones function similarly to the corresponding wild-type controls (Fig. 3, *middle right panel*). At present, we cannot tell if the low level of expression was idiosyncratic to this experiment or represents a significant trend. However, it is worth noting that the AC33 mutation eliminates a pair of conserved cysteine residues that are likely to be palmitoylated (17), and mutation of a palmitoylated cysteine in the 67-residue cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor decreases the half-life and impairs the function of this receptor (18, 19). While it is clear that the AC33 construct can partially correct secretion of lysosomal enzymes, further study is required to determine the functional significance of this region.

Three additional mutants are classified as borderline-impaired (AC20, E = 0.65 ± 0.13; AC28, E = 0.73 ± 0.17; and AC37, E = 0.69 ± 0.11). This assignment is supported by inspection of the scattergrams (Fig. 3): while increased expression of each construct partially corrects secretion of lysosomal enzymes, most clones exhibit higher secretion compared with clones containing similar levels of wild-type receptor. The AC20 mutation changes ^{74}LSS^{76} to alanine; the AC28 mutation changes ^{40}TEWL^{43} to alanine. These regions have not yet been further dissected to determine which side chains are important for sorting. The AC37 mutation eliminates four positive charges in the juxtamembrane region of the cytoplasmic domain (^{1}KKERR^5 changed to ^{1}AAEAA^5). To investigate this region further, we analyzed two mutants in which either two lysines or two arginines in this region were replaced with alanines. Neither mutant was markedly different compared with the normal receptor (Fig. 3 (*bottom right panel*) and Table I). It is noteworthy that these residues are part of a consensus stop transfer sequence found on many other membrane proteins (20). Thus, the positively charged cluster may be important for correct positioning of the cytoplasmic domain with respect to the membrane rather than being part of the sorting signal *per se*.

Three impaired mutants encompass the last 12 amino acids of the 166-residue cytoplasmic domain (AC01, AC02, and AC03) (Fig. 4). AC01 and AC02 are clearly impaired as judged from their sorting efficiencies and inspection of the scattergrams. For AC03, in the course of five independent transfection experiments using two different constructs encoding the same amino acid changes, almost all clones isolated contained low levels of receptor. The two experiments shown (Fig. 4 (*bottom left panel*) and Table I) represent the highest receptor levels found, and here, the individual clones containing medium (0.28 ng/μg) and high (1.06 ng/μg, plotted on the *right axis*) levels of receptor are impaired.

These findings are in good agreement with our previous findings (8), where alanine scanning of the C-terminal 14 residues revealed that the consensus casein kinase II site followed by two leucines (^{157}DDSDEDLL^{164}) is important. A second sequence that contains a consensus casein kinase II site followed by two hydrophobic residues (^{80}DDQDSEDEVL^{89}) occurs in the middle of the cytoplasmic domain, and both casein kinase II sites are phosphorylated *in vivo* (21–23). However, this internal sequence is not required for efficient lysosomal enzyme sorting as the three alanine cluster mutants that span residues 80–91 are all indistinguishable from the wild-type receptor (Fig. 4, *bottom right panel*). Taken together, these results indicate that most of the cytoplasmic domain is replaceable, and with the possible exception of the tyrosine-based

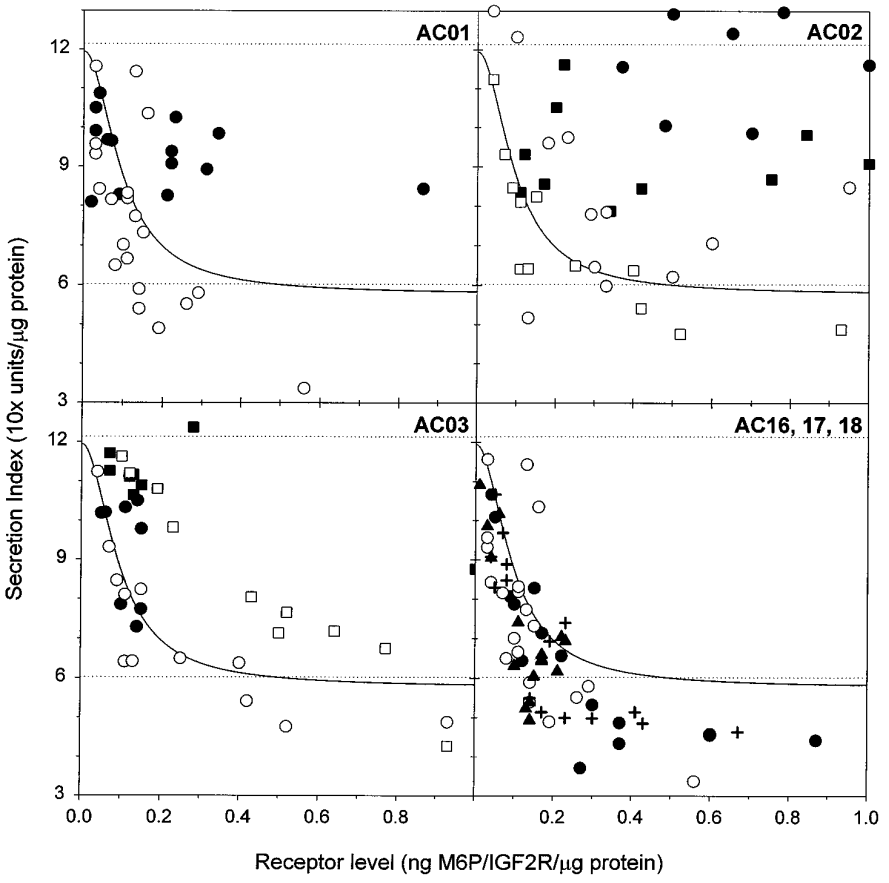

FIG. 4. **Effect of alanine cluster mutations that encompass the two casein kinase II sites.** Data are plotted as described in the legend to Fig. 3. *Top right panel*, *circles* represent data from Experiment 330, and *squares* represent data from Experiment 359. *Bottom left panel*, *circles* represent data from Experiment 359, and *squares* represent data from Experiment 375. *Bottom right panel*, *closed circles* are AC16, *plus signs* are AC17, and *closed triangles* are AC18.

internalization signal, the C-terminal region of the receptor contains the major determinant for lysosomal enzyme sorting.

Positional Effects on the C-terminal Sorting Signal

To further investigate the C-terminal sorting signal, we created a series of deletion and insertion mutants. In our previous study (8), alanine substitution of either His-165 or Ile-166 had no effect on receptor function, while mutations of upstream residues had marked effects. Interestingly, deletion of the C-terminal two residues clearly impaired receptor function (E = 0.53 ± 0.20) (Fig. 5, *top right panel*). The sorting efficiency of this mutant was only slightly higher than that of constructs containing deletions of the C-terminal 4, 13, 17, or 53 residues (representative scattergrams in Fig. 5 (*top left panel*) and Table I). The C terminus was extended by insertion of two alanines between Leu-164 and His-165 (construct LL(+AA)HI). Despite the nearly normal sorting efficiency (E = 0.86 ± 0.26), this mutation may have a modest effect on receptor function in sorting: while two clones containing high levels (1.01 and 1.36 ng/μg) appear normal, most clones had higher secretion indices than clones containing equivalent levels of the wild-type receptor (Fig. 5, *bottom left panel*). Addition of three alanines to the C terminus clearly impaired sorting (LLHI(+AAA), E = 0.61 ± 0.07), while addition of one alanine had little effect (LLHI(+A), E = 0.97 ± 0.10) (Fig. 5, *bottom right panel*). These results suggest that the precise distance of the C-terminal carboxylate from the sorting signal is not critical; rather, downstream residues are important for proper presentation of the determinant.

Given the similarity between the two casein kinase II sites of the receptor, it is possible that if presented in a different context, the internal casein kinase II site could be converted into a functional sorting signal. To test this hypothesis, we deleted an internal fragment of the cytoplasmic domain (residues 88–162) to produce the mutant Δ79(+LLHI), which had the C-terminal sequence ^{80}DDQDSEDELLHI^{91}. This mutant was clearly impaired in sorting (Fig. 5, *top right panel*), indicating that either spacing or other specific features of the normal C-terminal signal (see below) are required for efficient function in sorting.

Dissection of the C-terminal Sorting Signal

The major sorting determinant of the M6P/IGF2R contains two features of potential functional significance. First, the region contains two adjacent leucines, and dileucine motifs are putative signals for intracellular trafficking of proteins. Second, the serine and surrounding acidic cluster form an almost perfect consensus casein kinase II site (24), which has been shown to be phosphorylated *in vivo* (21–23). To dissect the key features of this region, we conducted a high resolution mutagenesis analysis of these residues. The results of this analysis, combined with our previous data, are summarized in Fig. 6.

Dileucine Motif—In our previous study (8), we found that alanine substitution of either Leu-163 or Leu-164 had marked effects on sorting. To further investigate their role, we replaced each residue with valine and isoleucine. Only low expressing clones were isolated for the L164V mutant, precluding evaluation of this substitution (data not shown). The L163V mutant had a borderline sorting efficiency (E = 0.72 ± 0.06) and appeared impaired relative to the normal receptor (Fig. 7, *top left panel*). In contrast, the two isoleucine replacement mutants appeared to function normally (Fig. 7, *top panels*). Taken together with the alanine scanning mutants, these data stress the importance of large aliphatic side chains at these two positions.

Casein Kinase II Site—We previously found that replacing Ser-159 with alanine impairs sorting (8). In contrast, Johnson and Kornfeld (9) analyzed a single clone that expressed a

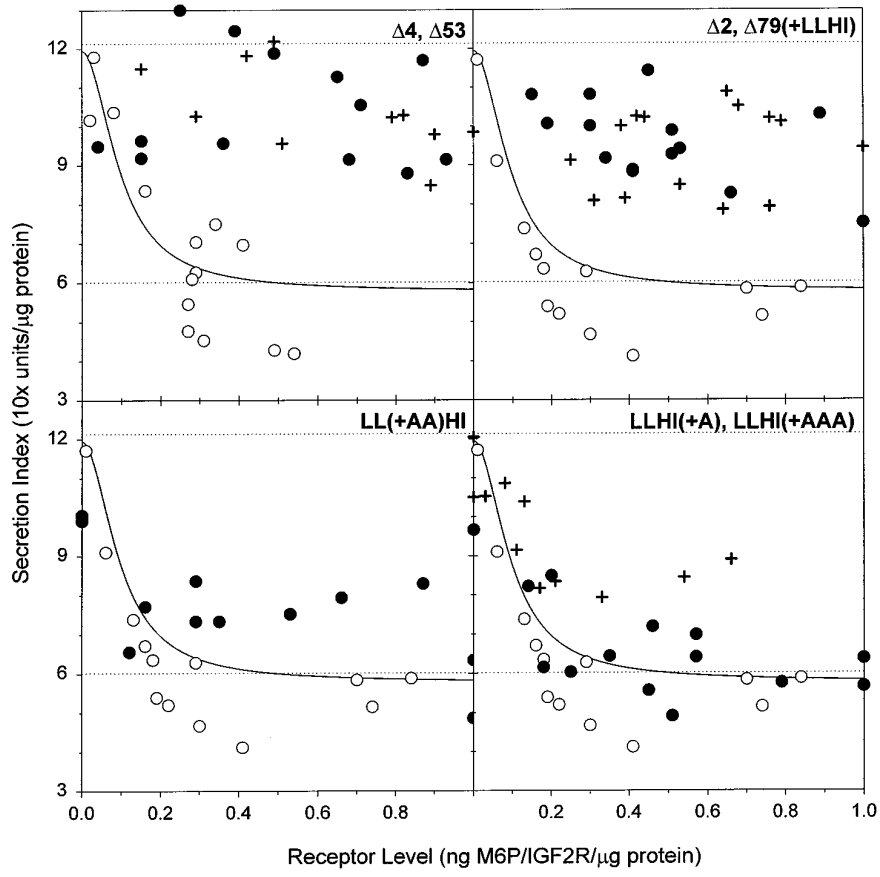

FIG. 5. **Effect of deletion and insertion mutations.** Data are plotted as described in the legend to Fig. 3. *Top left panel*: *closed circles*, Δ4; *plus signs*; Δ53. *Top right panel*: *closed circles*, Δ2; *plus signs*, Δ79(+LLHI). *Bottom right panel*: *closed circles*, LLHI(+A); *plus signs*, LLHI(+AAA).

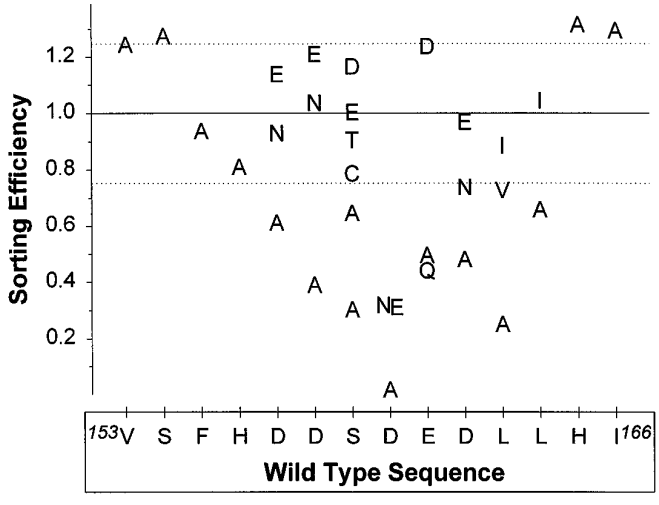

FIG. 6. **Summary of the mutational analysis of the C-terminal sorting signal.** The calculated sorting efficiencies of the mutants analyzed in this study are taken from Table I. The *solid* and *dotted reference lines* mark the mean ± 2 S.D. for the wild-type receptor. Sorting efficiencies of the alanine scanning mutants analyzed in our previous study (8) were calculated using the positive and negative controls for that data set.

mutant bovine receptor with multiple alanine substitutions at potential kinase sites (murine numbering, S19A, T40A, S84A, and S159A) and found that it functioned normally in terms of targeting cathepsin D. To recheck our findings, we confirmed the cytoplasmic domain sequence of the S159A mutant and conducted an independent transfection experiment to assess its function in sorting. These results corroborate our original observations, verifying that in our system, the receptor does not readily tolerate alanine substitution at position 159 (Table I and data not shown).

To examine the role of Ser-159 in sorting, we replaced this residue with threonine, cysteine, glutamate, or aspartate. None of the mutations had major effects on sorting (Fig. 7 (*bottom panels*) and Table I). However, from the sorting efficiencies and inspection of the individual scattergrams, it appears that the S159D mutant may be marginally more efficient than the normal receptor, and the S159C mutant may be slightly impaired. Taken together with the alanine scan, these results indicate that a polar side chain at position 159 is important, but show that cycles of serine phosphorylation/dephosphorylation are not obligatory for intracellular trafficking of the M6P/IGF2R.

We previously found from alanine scanning mutagenesis that each of the five charged residues flanking Ser-159 was important for efficient sorting (8). To determine if charge or shape was important, we examined the effects of isoelectric and isosteric substitutions. Four residues readily tolerated conservative substitutions: mutants with isoelectric substitutions at Asp-157 (D157E), Asp-158 (D158E), Glu-161 (E161D), and Asp-162 (D162E) all functioned at least as efficiently as the wild-type receptor (Fig. 8 and Table I). Isosteric substitutions at some of these residues were relatively innocuous, with the D162N, D158N, and D157N mutants functioning similarly to the wild-type receptor (Fig. 8 and Table I; also data not shown). The E161Q construct had a low sorting efficiency (E = 0.43 ± 0.27). While this may have been a result of the general low expression, comparison of E161Q clones containing similar levels of receptor with both the E161D mutant and positive control suggests that the isosteric mutation is not well tolerated (Fig. 8, *bottom left panel*). Asp-160 was unique in that isoelectric and isosteric substitutions severely impaired function (D160E, E = 0.30 ± 0.13; and D160N, E = 0.31 ± 0.18) (Fig. 8, *top right panel*), indicating that this residue is a critical component of the sorting signal.

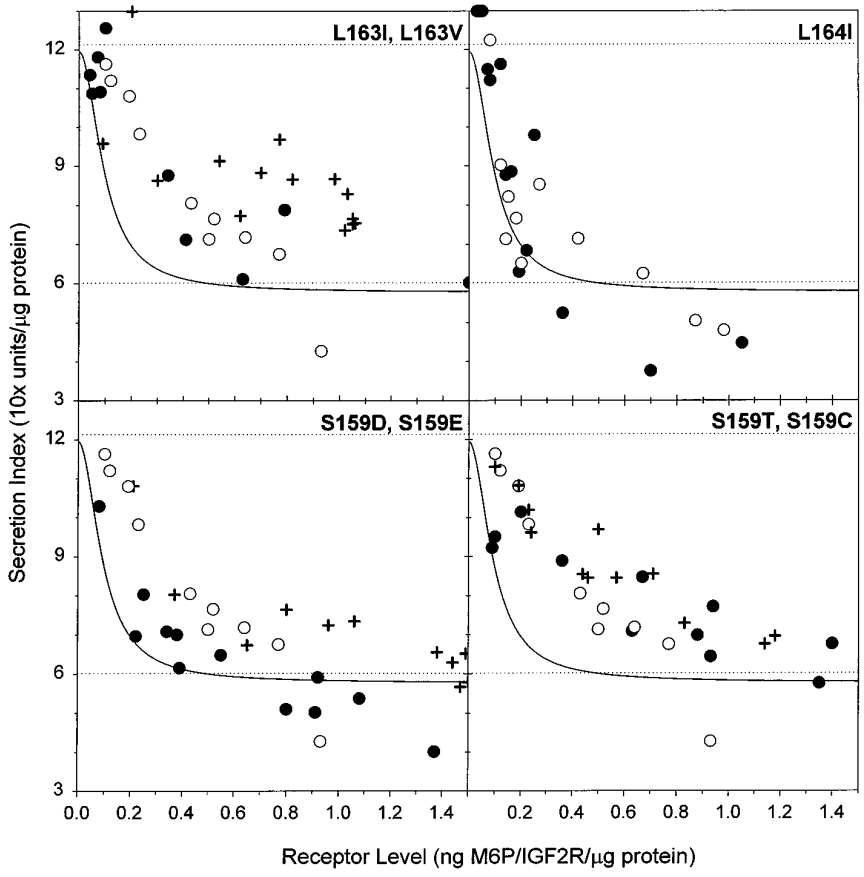

FIG. 7. **Effect of mutations on the dileucine motif and Ser-159.** Data are plotted as described in the legend to Fig. 3. *Top left panel*: *closed circles*, L163I; *plus signs*, L163V. *Bottom left panel*: *closed circles*, S159D; *plus signs*, S159E. *Bottom right panel*: *closed circles*, S159T; *plus signs*, S159C.

Sorting Mutants Have Normal Endocytic Efficiency and Surface Distributions

One concern is that mutations potentially could increase the rate at which the receptor traveled to the plasma membrane or decrease the rate of internalization. This could result in depletion of the intracellular pool of the receptor and thus indirectly interfere with sorting. In the course of selecting clonal cell lines for this study, all clones were first screened by immunofluorescence to estimate relative receptor expression and to evaluate clonal homogeneity as described previously (8). No marked differences in cellular distribution were evident. As an additional quantitative test, we measured the surface distribution of one or two clones for all constructs analyzed in this study. Here, intact and saponin-permeabilized cells were probed with ^{125}I-labeled anti-M6P/IGF2R monoclonal antibody to estimate cell-surface and total receptor, respectively. For the wild-type receptor, 91–96% was intracellular as determined by analysis of six different clones. Control experiments revealed that a truncated bovine M6P/IGF2R with a seven-residue-long cytoplasmic domain that lacks the tyrosine internalization signal (cell line Dd4 (5)) accumulated on the cell surface, with only 44% being intracellular. In contrast, all the mutants described in this and our previous (8) studies were at least 85% intracellular (compiled in Ref. 15; data not shown). This indicates that the intracellular pool of the receptor was not sufficiently depleted to interfere with sorting.

We also measured the endocytic efficiency of a number of the highly impaired sorting mutants. These experiments were carried out essentially as described (6). Briefly, cells were placed on ice to arrest endocytosis, incubated with ^{125}I-labeled β-glucuronidase at 4 °C for 1 h, washed, and rapidly warmed to 37 °C, and the radioactivity released into the medium, bound to the surface, and internalized was measured at different time intervals. The normal receptor internalized ligand rapidly, with a half-time of ~1 min (Fig. 9, *top left panel*). In contrast, the control Dd4 cell line was markedly impaired (Fig. 9, *bottom right panel*). For all other mutants analyzed, the significant finding was that while minor differences may be present, they internalized ligand at rates comparable to the normal receptor (see Fig. 9 and legend). Thus, these severely impaired sorting mutants fold properly as demonstrated by their ability to bind and rapidly internalize ligand.

DISCUSSION

One noteworthy finding of this study is that apart from the endocytosis signal, a single contiguous stretch of eight residues near the C terminus is the sole major sorting determinant revealed by our assay. While part of the cytoplasmic domain may be structural, additional determinants may be important for other processes such as proper trafficking in polarized cells (25, 26) or insulin-stimulated redistribution to the cell surface in adipocytes (27). In addition, it is possible that regions outside the C-terminal region not detected in our assay contribute to sorting. For instance, if the concentration of sorting components and their affinity for the receptor cytoplasmic domain are sufficiently high, then mutations that cause modest changes in affinity may not be apparent. This could be exacerbated if the host cell line has up-regulated other components of the sorting system in an attempt to compensate for impaired lysosomal transport. Nonetheless, our results clearly demonstrate that the C-terminal region is of paramount importance for sorting.

The C-terminal region of the receptor contains two recognizable sequence elements, a dileucine motif and a consensus casein kinase II site. As summarized in Fig. 6, this and our previous (8) studies demonstrate that both elements are involved in sorting. At present, we cannot distinguish whether

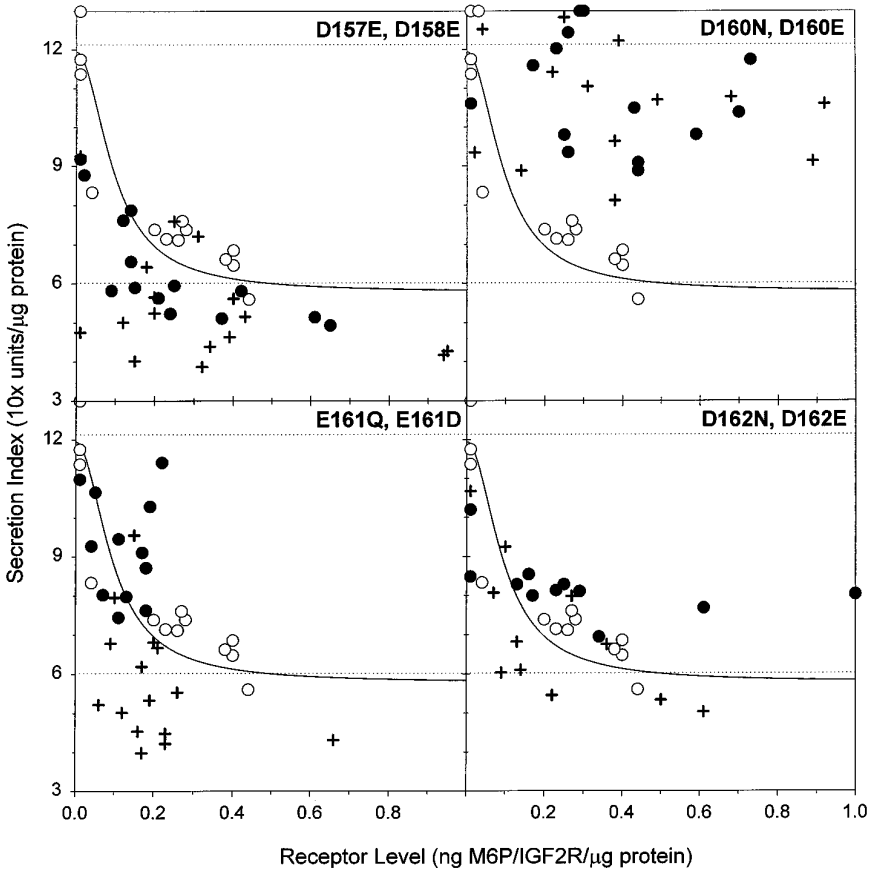

FIG. 8. **Effect of isosteric and isoelectric substitutions at acidic residues within the C-terminal casein kinase II site.** Data are plotted as described in the legend to Fig. 3. *Top left panel*: *closed circles*, D157E; *plus signs*; D158E. *Top right panel*: *closed circles*, D160N; *plus signs*, D160E. *Bottom left panel*: *closed circles*, E161Q; *plus signs*, E161D. *Bottom right panel*: *closed circles*, D162N; *plus signs*, D162E.

both or one of these elements is involved in either departure from the *trans*-Golgi network to endosomes or transit from endosomes to the *trans*-Golgi network. However, recent studies on the cation-dependent mannose 6-phosphate receptor, which also has a consensus casein kinase II site and dileucine motif near the C terminus (EESEERDDHLLPM), suggest that these motifs may have independent functions: mutations in either motif alone resulted in impaired sorting, while only mutations in the consensus casein kinase II site impaired binding of the Golgi adaptor AP1 in an *in vitro* assay (28).

Dileucine Motif

Dileucine motifs are thought to participate in a wide range of targeting steps. This sequence was first noted as a semi-autonomous targeting element on the cytoplasmic domain of the CD3 γ- or ϵ-subunit that could direct a Tac antigen chimera to the lysosome (29). Depending on the presence or absence of an intact tyrosine signal, this determinant could mediate direct (Golgi to lysosome) or indirect (Golgi to surface to lysosome) targeting. Subsequently, dileucine or dihydrophobic motifs have been implicated in trafficking events including lysosomal targeting of lysosomal integral membrane protein II (30, 31), endocytosis and basolateral sorting of macrophage IgG Fc receptors (32, 33), trafficking of the Glut4 glucose transporter (34–36), rapid internalization of the major histocompatibility complex invariant chain (37), internalization of the insulin receptor (38), protein kinase C-dependent down-regulation of the T cell receptor (39), as well as intracellular trafficking of both types of mannose 6-phosphate receptors (8, 9, 28, 40). While in some of these cases perturbations of adjacent hydrophobic residues may have indirect effects on another functional element, experiments with reporter molecules support the notion that the dileucine-based determinants represent trafficking signals.

The information content of dileucine or dihydrophobic motifs seems rather low, as given their relatively high abundance, they are commonly found in the cytoplasmic regions of many transmembrane proteins. (From analyzing sequences in the SwissProt data base (Version 14), the frequency of finding two adjacent residues being leucine is 0.9%; being leucine or isoleucine, 2.3%; and being leucine, isoleucine, or valine, 4.7%. The probability (p) of a random sequence of n residues containing one or more pairs of adjacent residues with a given abundance (f) is $p = 1 - (1 - f)^{n-1}$. Thus, for a 100-residue sequence, the probability of having adjacent leucines or isoleucines is ~90%.) The wide variety of sequences that function in reporter constructs suggests that if these elements are recognized by a common binding site, the requirements for molecular recognition are rather loose. In this respect, dileucine motifs resemble tyrosine motifs, where a wide variety of related sequences can direct internalization from the plasma membrane, albeit at vastly different rates (41).

One intriguing possibility is that dileucine motifs evolved from a quality control pathway that serves to target damaged membrane proteins to the lysosome. In this case, protein damage would cause the normally inaccessible dihydrophobic determinant to be exposed at the surface of the molecule. If there were no downstream retrieval event, this pathway could function with relatively slow kinetics, as the targeting step would be essentially irreversible. Relatively inefficient signals may also suffice for delivery of resident membrane proteins to lysosomes. At the other end of the spectrum, receptors such as the M6P/IGF2R whose function requires rapid recycling between different compartments may require more efficient signals, and the recognition determinant may be more extensive. It will be important to identify the cellular components that recognize these motifs and to investigate their role in both trafficking and protein turnover.

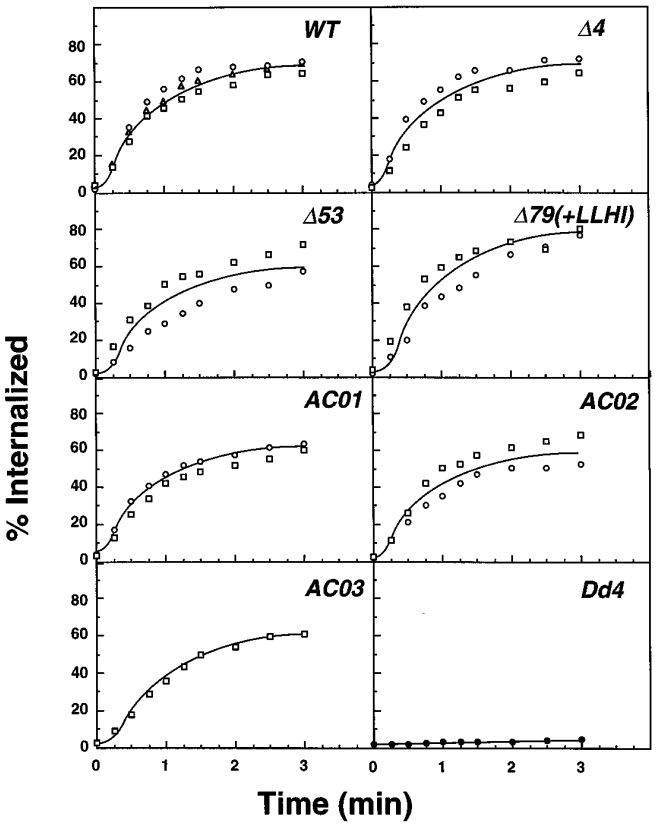

FIG. 9. **Effect of mutations on M6P/IGF2R internalization.** Cells were evaluated for their ability to internalize prebound ^{125}I-labeled β-glucuronidase as described under "Experimental Procedures." In each panel, the different symbols represent analysis of individual clonal cell lines expressing the indicated receptor construct and are the average of two independent experiments. The endocytic rate constants (mean ± S.D.) for each clone analyzed are calculated as described under "Experimental Procedures" and are given in units of min^{-1}: wild-type (*WT*) receptor, 0.61 ± 0.09, 0.74 ± 0.02, 0.66 ± 0.01; Δ4, 0.60 ± 0.19, 0.84 ± 0.12; Δ53, 0.63 ± 0.17, 0.44 ± 0.24; Δ79(+LLHI), 0.89 ± 0.02, 0.62 ± 0.06; AC01, 0.57 ± 0.15, 0.65 ± 0.04; AC02, 0.66 ± 0.01, 0.55 ± 0.09; AC03, 0.44 ± 0.04; and Dd4, 0.010 ± 0.001.

Consensus Casein Kinase II Site

Serine Phosphorylation Is Not Essential for Function—Studies by Hoflack and co-workers (21, 22) suggest that phosphorylation of the casein kinase II sites in the cytoplasmic domain may be involved in receptor trafficking. In experiments performed on normal rat kidney cells, both Ser-159 and Ser-84 underwent cycles of phosphorylation/dephosphorylation, and phosphorylation occurred either in the *trans*-Golgi network or shortly after exit. Interestingly, while the distribution of the data obtained from our experimental system precludes firm conclusions regarding minor functional differences, close inspection of the data suggests that the rank order for intrinsic sorting efficiency may be S159A < S159C < S159T ~ wild-type ~ S159E < S159D (Figs. 6 and 7). As aspartate can mimic phosphoserine in poly-Ig receptor trafficking (42), and threonine can serve as a substrate for casein kinase II (24), it is possible that phosphorylation may increase the affinity of the receptor for cellular components that effect trafficking. This would be consistent with *in vitro* binding studies (43). However, an important finding is that in our experimental system, cycles of phosphorylation/dephosphorylation clearly are not obligatory for receptor function in sorting.

The Acidic Cluster and Aspartate 160 Are Critical Components of the Sorting Signal—From the analysis of Ser-159 and the surrounding residues, we conclude that the acidic cluster, rather than the serine itself, is the most critical feature of the consensus casein kinase II site. Interestingly, acidic clusters have been noted to occur on the cytoplasmic domains of other cycling proteins (44). Recently, acidic clusters in the cytoplasmic domain of furin (45–48) and invariant chain (49) have been implicated in intracellular trafficking events. The acidic clusters may be important for protein-protein interactions, either through direct contact or by coordinating a divalent cation. Alternatively, the aspartate may have some key catalytic role or may be transiently modified, as found in the bacterial two-component system (50). It will be interesting to determine if key aspartates are also critical for the intracellular trafficking functions of other proteins and, if so, to further dissect their role in the trafficking process.

Acknowledgments—We thank Jill Remmler for technical assistance and Li Lin and David Sleat for critical reading of the manuscript.

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