A Novel Sorting Sequence in the $\beta_2$-Adrenergic Receptor Switches Recycling from Default to the Hrs-dependent Mechanism*

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Plasma membrane recycling of G protein-coupled receptors can occur by at least two distinct mechanisms as follows: a “default” mechanism that occurs nonselectively, and a specifically sorted mechanism that requires the endosome-associated protein Hrs. In this study we have defined a sequence in the $\beta_2$-adrenergic receptor cytoplasmic tail that confers Hrs dependence on receptor recycling. This sequence resembles acidic dileucine class motifs found in other membrane proteins but is structurally and functionally distinct from previously identified sorting sequences. Mutation of the novel sorting sequence rendered plasma membrane recycling independent of Hrs and independent of a distal PDZ ligand required for Hrs-dependent recycling. We propose that the novel sorting sequence functions to “switch” endocytic trafficking between mechanistically distinct recycling modes, thereby explaining failure of the wild type $\beta_2$-adrenergic receptor to recycle efficiently by default.

Many G protein-coupled receptors (GPCRs) undergo ligand-induced endocytosis to a common population of early endosomes, from which receptors are sorted to divergent pathways in a highly specific manner (1–3). Sorting of endocytosed receptors to lysosomes contributes to down-regulation of receptor number and prolonged attenuation of cellular signaling. In contrast, recycling of endocytosed receptors to the plasma membrane restores surface receptor number and promotes rapid functional recovery (resensitization) of cellular responsiveness to ligands (2, 4).

There is increasing evidence that a number of GPCRs require specific structural determinant(s) within their cytoplasmic domains in order to undergo efficient recycling to the plasma membrane (5–11). This process of “sequence-directed” recycling differs from “default” recycling of many other integral membrane proteins, such as the transferrin receptor, which occurs in the absence of any known cytoplasmic determinant and apparently by bulk membrane flow (12, 13). Sequence-directed recycling has been studied in some detail for the $\beta_2$-adrenergic receptor ($\beta_2$ADR), a GPCR for which the physiological importance of this membrane pathway has been particularly well established (4, 14).

An early clue to the existence of the specialized recycling mechanism was the observation that efficient recycling of the $\beta_2$ADR requires a C-terminal sequence conforming to a type 1 PDZ ligand that also binds the N-ethylmaleimide-sensitive factor. Mutation of this “recycling signal,” or disruption of cytoplasmic protein interactions with this domain, profoundly impairs recycling of the $\beta_2$ADR (5–7, 15).

We have recently demonstrated another fundamental distinction between default and sequence-directed recycling. The endosome-associating protein hepatocyte-growth factor substrate (Hrs) is essential for sequence-directed recycling of the $\beta_2$ADR (16), in addition to the previously established role of Hrs in targeting membrane cargo to lysosomes for degradation (17). In contrast, receptors that recycle by a default mechanism do not require Hrs, as shown for the transferrin receptor and a truncated mutant V2 vasopressin receptor, V2R 362T (16, 18, 19). Therefore, we proposed a model where Hrs controls sequence-directed recycling of receptors through shared endocytic compartments mediating default recycling of other membrane cargo (16).

This model raises a primary issue in understanding how entry into this specialized recycling pathway occurs. Mutation of the C-terminal PDZ ligand (5–7, 15), as well as depletion of cellular Hrs (16), strongly inhibits recycling of internalized receptors. Remarkably, neither of these manipulations produce default recycling of receptors, which is the trafficking phenotype expected in the absence of endocytic sorting signals (12). These considerations lead to the question of whether the $\beta_2$ADR contains additional sorting information, which directs endocytosed receptors to traffic via the Hrs-dependent mechanism and effectively prevents receptor recycling by default.

In this study we identify a novel sorting sequence, present in a conserved region of the $\beta_2$ADR cytoplasmic tail, that “switches” the mode of receptor recycling from default to Hrs-dependent mechanisms. These results provide fundamental insight to the regulated membrane trafficking of a prototypic GPCR and reveal a new type of endocytic sorting sequence.
Novel Sorting Sequence Regulates Hrs-dependent Recycling

EXPERIMENTAL PROCEDURES

Materials—Isoproterenol and alprenolol were obtained from Sigma. [Arg8]Vasopressin was obtained from Bachem.

Eukaryotic Expression Constructs and siRNA Oligonucleotides—FLAG-human β2-adrenergic receptor (β2ADR) and FLAG-human V2 vasopressin truncation mutant (V2R 362T) have been described previously (5, 20). The Myc-tagged Hrs in pcDNA3 was a gift from Harald Stenmark (Norwegian Radium Hospital, Norway). The HA-tagged chimera of V2R/β2ADR (340–413) was a gift from Marc Caron (Duke University, Durham, NC) (21). The V2R 362/β2ADR-(366–413) chimera was constructed by introducing an EcoRV site in the full-length V2R at residue 362 by site-directed mutagenesis (QuikChange®, Stratagene). The mutated V2R was digested with EcoRV/XbaI and ligated with a β2ADR C-tail fragment (residues 366–413) produced by digestion of full-length β2ADR with EcoRV and XbaI (β2ADR has an endogenous EcoRV site at residue 366). V2R 362/β2ADR-(410–413) and -(404–413) were created by ligation of a linker corresponding to the last 4 or 10 amino acid residues of the β2ADR (flanked by EcoRV and XhoI overhangs), with EcoRV/XhoI-digested V2R as above. Acidic dileucine sequences in full-length β2ADR and V2R 362/β2ADR chimeras were mutated to alanine by site-directed mutagenesis (QuikChange, Stratagene). Addition of C-terminal alanine to V2R 362/β2ADR chimeras was carried out by site-directed mutagenesis. All constructs were confirmed by dideoxynucleotide sequencing.

Control and Hrs siRNA duplex RNA oligonucleotides (Qiagen) were used as described previously and validated (16, 22, 23).

Cell Culture and Transfection—HeLa cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, glutamine (0.3 mg/ml), and penicillin/streptomycin (100 units/ml) at 37 °C in 5% CO2. Transient transfections were performed with Lipofectamine 2000 (Invitrogen), and cells were assayed 24 h post-transfection except for siRNA transfections, where cells were assayed 72 h post-transfection.

Immunofluorescence Staining and Confocal Imaging—Visualization of FLAG receptors and myc-Hrs was carried out using indirect immunofluorescence microscopy of HeLa cells. Surface receptors were labeled by exposing intact cells to rabbit anti-FLAG antibody (1:1000, Sigma) for 20 min at 37 °C, prior to incubation for an additional 20 min in the absence (untreated condition) or presence of the appropriate agonist (10 μM isoproterenol or AVP) to promote endocytosis of labeled receptors. Cells were then washed extensively and further incubated in media for 1 h (agonist washout condition) to allow for recycling. To visualize PDZ-dependent recycling of internalized receptors, FLAG-tagged receptors were fed with mouse anti-FLAG M1 antibody (1:1000, 20 min; Sigma) at 37 °C, prior to agonist stimulation as above. Agonist-treated cells were washed four times in PBS, 0.04% EDTA to selectively remove antibody bound to residual surface receptors, and cells were then incubated in agonist-free media for 60 min at 37 °C. For all experiments, cells were fixed using 4% paraformaldehyde freshly dissolved in PBS, incubated in blocking solution (PBS plus 1% bovine serum albumin and 10% goat serum, pH 7.4), and permeabilized with 0.1% Triton X-100. For myc-Hrs-expressing cells, mouse anti-Myc antibody (1:500; Upstate) was added in blocking solution for 60 min. Specimens were washed in PBS and then incubated for 30 min with goat anti-mouse Texas Red and donkey anti-rabbit Alexa Fluor 488-conjugated antibodies (1:1000, Molecular Probes) in blocking solution. Specimens were washed extensively in PBS, mounted on glass slides, and examined using a Zeiss LSM510 confocal laser microscope with an oil immersion 63x/NA1.3 objective, using instrument settings verified to produce negligible bleed through between channels and an estimated section thickness of 1 μm. Micrographs shown are representative optical sections imaged through the center of the cell.

Flow Cytometry—Fluorescence flow cytometry was used to quantitate internalization and recycling of receptors by measuring levels of cell surface FLAG-tagged receptor as described previously (16). Briefly, adherent cells were surface-labeled with anti-FLAG or anti-HA (for V2R/β2ADR-(340–413)) antibody (20 min, 37 °C) prior to incubation in the presence (untreated condition) or presence of the appropriate agonist (10 μM isoproterenol or AVP, 20 min). Cells were chilled on ice to stop subsequent receptor trafficking (agonist-treated condition) or washed and subsequently incubated at 37 °C in the absence of agonist for the indicated time period (washout condition). Cells were then lifted with 0.05% trypsin/PBS, washed with ice-cold PBS, 2% fetal calf serum, and incubated for 40 min on ice with anti-mouse secondary antibodies conjugated to either phycoerythrin (1:50) (Sigma) or Alexa Fluor 488 (1:500) (Molecular Probes). Fluorescence intensity profiles of cell populations (10,000 cells/sample) were measured using a FACSCalibur instrument (BD Biosciences). In each experiment, duplicate treatments were analyzed for each condition. All experiments were carried out at least in triplicate (number indicated in figure legends), and values reported were derived from the mean determination across experiments. The percentage of receptor recycling was calculated from mean surface receptor fluorescence values (F) as follows: % recycling = (Fwashout − Fagonist-treated)/(Funtreated − Fagonist-treated) × 100.

Western Blotting—Immunoblotting to detect total cellular levels of Hrs was carried out as described previously (16, 23). Briefly, cells were washed three times in ice-cold phosphate-buffered saline (PBS) and lysed in extraction buffer (0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 25 mM Tris, pH 7.4, 1 mM CaCl2, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation (12,000 × g for 30 min) and then mixed with SDS sample buffer for denaturation. The proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed for Hrs (1:2000; Alexis Biochemicals) or glyceraldehyde-3-phosphate dehydrogenase (1:40,000; Chemicon) by immunoblotting using horseradish peroxidase-conjugated sheep anti-mouse IgG (1:5000; Amersham Biosciences) and SuperSignal detection reagent (Pierce).
Statistical Analysis—Statistical significance was determined using paired Student’s t test. Differences were considered significant at $p < 0.05$.

RESULTS

The C-terminal PDZ Ligand Present in the $\beta_2$ADR Cytoplasmic Tail Is Not Fully Sufficient to Confer Hrs-dependent Recycling on a Chimeric Mutant Vasopressin Receptor—Previous studies defined a minimal recycling signal, contained within the distal 4–10 residues of the $\beta_2$ADR C-terminal tail (C-tail), which is necessary for efficient recycling of the $\beta_2$ADR and sufficient to re-route a distinct GPCR from lysosomal to recycling pathways (5–7, 15). We recently showed that a sequence corresponding to residues 366–413 of the $\beta_2$ADR C-tail, which includes these distal residues, is sufficient to confer Hrs-dependent recycling when fused to a truncated mutant V2 vasopressin receptor (V2R 362T) that otherwise recycles by default and in an Hrs-independent manner (16, 20, 24). Thus we asked whether the distal recycling signal is sufficient, by itself, to confer Hrs dependence on V2R 362T recycling. To begin to address this question, we examined the endocytic trafficking of a series of functional V2R/$\beta_2$ADR chimeras and took advantage of the fact that overexpression of a Myc-tagged Hrs construct (myc-Hrs) acts as a dominant negative to selectively inhibit Hrs-dependent recycling of receptors (16).

All chimeric receptors tested exhibited rapid agonist-induced internalization, which was evident qualitatively by confocal microscopy (not shown) and quantified by fluorescence flow cytometric analysis of decreased surface receptor fluorescence produced by 20 min of exposure to 10 $\mu$M of the
agonist peptide AVP (Fig. 1A, open bars). Overexpression of myc-Hrs did not detectably affect AVP-induced internalization of any of the mutant receptors (Fig. 1A, solid bars). All of the mutant receptors also recycled efficiently after internalization, as indicated by recovery of surface receptor fluorescence measured 60 min after agonist washout (Fig. 1B, open bars). myc-Hrs overexpression (Fig. 1B, solid bars) did not affect plasma membrane recycling of the V2R 362T (Fig. 1B, compare 1st and 2nd bars) but strongly inhibited recycling of the V2R 362/362ADR-(366–413) chimeric mutant receptor (Fig. 1B, 3rd and 4th bars), as shown previously (16). Another chimeric receptor, which contains essentially the entire C-tail of the β2ADR (residues 340–413) and has been shown to efficiently recycle (21), also exhibited recycling that was strongly inhibited by overexpressed myc-Hrs (Fig. 1B, 5th and 6th bars). Together these results confirm the Hrs-independent nature of V2R 362T mutant receptor recycling and the ability of structural determinant(s) located within the β2ADR C-tail to confer Hrs dependence on receptor recycling (16).

Despite the ability of β2ADR-derived tail sequence to confer Hrs sensitivity on recycling of both mutant receptors, we were surprised to observe that neither the distal 4 nor 10 C-terminal residues of the β2ADR tail (residues 410–413 and 404–413) themselves conferred detectable Hrs sensitivity on mutant receptor recycling (Fig. 1B, 7th to 10th bars). This pronounced difference in Hrs sensitivity of recycling was also evident by microscopic localization of chimeric receptors, where myc-Hrs expression was verified directly in the same cells by dual-label immunocytochemistry (Fig. 1C). In the absence of myc-Hrs expression, these mutant receptors recycled to the plasma membrane following agonist-induced internalization (Fig. 1C, top panels). In cells expressing myc-Hrs, V2R 362T mutant receptors returned to a predominantly plasma membrane localization after agonist washout (Fig. 1C, left column of images), consistent with Hrs-independent recycling of this receptor shown previously (16). V2R 362/362ADR-(340–413) mutant receptors remained localized in Hrs-associated endosomes, and little receptor recovery in the plasma membrane was visualized after agonist washout (Fig. 1C, middle column) as shown previously (16). In contrast, V2R 362/362ADR-(410–413) mutant receptors (as well as V2R 362/362ADR-(404–413) mutant receptors, not shown) returned to a predominantly plasma membrane localization after agonist washout in cells expressing myc-Hrs (Fig. 1C, right column). These results indicate that the distal recycling signal in the β2ADR C-tail is not sufficient to direct receptor trafficking from a default to Hrs-dependent recycling mechanism. They also suggest that an additional structural determinant, which mediates receptor sorting via the Hrs-dependent mechanism, is encoded within residues 366–404 of the β2ADR C-tail.

**Identification of a Distinct Sequence within the β2ADR C-tail That Confers Hrs-dependent Recycling on Endocytosed Receptors**—Inspection of the sequence corresponding to residues 366–404 of the β2ADR C-tail sequence revealed a dileucine flanked at −3 and −5 positions by acidic residues (EKENKLL, Fig. 2A). This sequence is similar to a family of acidic cluster-dileucine motifs implicated previously as a sorting signal in a number of membrane-trafficking processes, where both the leucines and upstream acidic residues are important for function (25). These key residues are conserved in the β2ADR across mammalian species, but no functional role of this conserved sequence has been identified previously. To begin to investigate a possible endocytic sorting function, the
corresponding residues were mutated to alanine (AKANKAA mutation, Fig. 2A), and effects on Hrs-dependent recycling were evaluated in the context of the V2R 362/β₂ADR-(366–413) chimera. HeLa cells were transfected with each construct, and trafficking was assessed by confocal microscopy and flow cytometry. The AKANKAA mutation did not detectably affect plasma membrane localization, agonist-induced internalization, or
Novel Sorting Sequence Regulates Hrs-dependent Recycling

A

% Receptor Internalized

WT AKANKAA LL/AA EKE/AKA LLCED/AACAA

β2-ADR

Control myc-Hrs

B

% Receptor Recycled

WT AKANKAA LL/AA EKE/AKA LLCED/AACAA

β2-ADR

Control myc-Hrs

C

control siRNA
Hrs siRNA
Hrs siRNA (2)

Hrs

GapDH

D

% Receptor Recycled

Control Control siRNA Hrs siRNA Hrs siRNA (2)

E

% Receptor Recycled

WT AKANKAA LL/AA EKE/AKA LLCED/AACAA

β2-ADR

Control Hrs siRNA

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recycling in cells expressing Hrs at endogenous levels (Fig. 2, B, top panels, and C, open bars, and data not shown). However, the AKANKAA mutation substantially reduced the ability of over-expressed myc-Hrs to inhibit receptor recycling. This was evident qualitatively by the ability of V2R 362/β3-ADR-(366–413)-AKANKAA mutant receptors to return to a plasma membrane localization pattern after agonist washout, in contrast to visible endosome retention of the V2R 362/β3-ADR-(366–413) receptor containing the wild type (EKENKLL) sequence (Fig. 2B).

The ability of the AKANKAA mutation to abrogate Hrs sensitivity of the chimeric receptor recycling was further verified quantitatively using the fluorescence flow cytometric assay (Fig. 2C, lower graph). Together, these findings suggest a possible functional role of the EKENKLL sequence in conferring Hrs dependence on GPCR recycling.

The Acidic Dileucine Sequence Is Also Required for Sequence-directed Recycling and Produces Default Recycling when Mutated—Because Hrs dependence appears to be a specific property of the sequence-directed recycling mechanism (16), we reasoned that the AKANKAA mutation might fully bypass this mechanism and thereby allow internalized receptors to recycle irrespective of the C-terminal PDZ ligand sequence essential for Hrs-dependent recycling of wild type receptors. To test this, we analyzed endocytic trafficking of the V2R 362/β2-ADR C-tail chimeras containing a single C-terminal alanine addition, which specifically disrupts the distal PDZ ligand required for recycling of wild type receptors (5, 7) when combined with the AKANKAA mutation of the acidic dileucine sequence. We also examined the effects of mutating either the dileucine or upstream acidic residues individually, and we further evaluated specificity by mutating downstream (rather than upstream) acidic residues (Fig. 3A).

First, we examined the effect of disrupting the distal PDZ ligand in a chimera that contains the intact acidic dileucine sequence (V2R 362/β2-ADR-(366–413)-Ala). Both confocal microscopy and quantitative flow cytometry indicated that disrupting the distal PDZ ligand strongly inhibited recycling of the chimeric mutant receptor after agonist washout (Fig. 3, B and C, compare WT and −Ala), consistent with the previous assignment of this sequence as an essential “recycling signal” in the β2-ADR tail. The inhibitory effect of the distal PDZ ligand disruption, however, was almost completely lost by simultaneous mutation of the acidic dileucine sequence. Confocal microscopy indicated that V2R 362/β2-ADR-(366–413)-AKANKAA-Ala mutant receptors returned to a predominantly plasma membrane localization after agonist washout (Fig. 3B, 3rd column of images). Furthermore, fluorescence flow cytometry indicated that V2R 362/β2-ADR-(366–413)-AKANKAA-Ala mutant receptors recycled to nearly the same extent as V2R 362/β-ADR-(366–413) receptors containing the wild type (WT) β2-ADR tail (Fig. 3C, compare 3rd and 1st bars). Moreover, V2R 362/β3-ADR-(404–413) chimeric mutant receptors, which lack the acidic dileucine sequence entirely, also recycled with similarly high efficiency irrespective of whether they contained an intact C-terminal PDZ ligand (WT) or if the PDZ ligand was disrupted (−Ala) (Fig. 3B, 7th and 8th columns, and Fig. 3C, 7th and 8th bars).

We next investigated the relative importance of the dileucine and upstream acidic residues in conferring PDZ dependence on receptor recycling. Alanine mutation of only the dileucine motif (LL/AA), or mutation of only the upstream acidic residues (EKE/AKA), in V2R 362/β2-ADR-(366–413)-Ala increased recycling over the V2R 362/β2-ADR-(366–413)-Ala chimera (Fig. 3B, 4th and 5th columns, and Fig. 3C, compare 2nd bar with 4th and 5th bars), suggesting that both the dileucine and upstream acidic residues contribute to the ability of the novel sorting sequence to confer PDZ dependence on receptor recycling. We further investigated specificity of this sequence by comparing the effect of mutating two downstream acidic residues together with the dileucine (AACAA mutation) with mutation of the two upstream acidic residues (AKANKAA) or the dileucine alone (LL/AA) (Fig. 3A). The “downstream” LLCED/AACAA mutation did not reduce PDZ dependence of receptor recycling over that of mutating the dileucine (LL/AA) by itself (Fig. 3B, 6th column, and Fig. 3C, compare 6th and 4th bars). Furthermore, the LLCED/AACAA mutation produced a much smaller effect on PDZ dependence than the “upstream” AKANKAA mutation, despite the closely similar local electrostatic effects expected from these mutations. Together these results support the biochemical specificity of the mutations tested and suggest that EKENKLL represents a core determinant conferring both Hrs and PDZ dependence on receptor recycling.

The Acidic Dileucine Sequence Is Required for Full-length β2-ADR Recycling via the Hrs-dependent Pathway—We next examined if the EKENKLL sequence functions in Hrs-dependent recycling of the full-length β2-ADR. Like V2R 362/β2-ADR-(366–413)-AKANKAA, mutation of the acidic dileucine motif in FLAG-tagged β2-ADR had no significant effect on surface localization, surface expression (89.5 ± 12.9% compared with wild type β2-ADR), agonist-induced internalization, or recycling of receptors in cells expressing Hrs at endogenous levels. This was established both by confocal microscopy and by flow cytometry assays (Fig. 4, A and B, white bars). Co-expression of myc-Hrs (dark bars) strongly inhibited recycling of the wild type β2-ADR (Fig. 4B, WT), as shown previously (16). However, this effect of myc-Hrs was markedly reduced by the acidic dileucine mutation (Fig. 4B, AKANKAA). The reduction in Hrs

**FIGURE 4.** Mutation of the EKENKLL sequence in the full-length β2-ADR results in default, Hrs-independent, recycling. A, HeLa cells expressing either FLAG-β2-ADR or mutant FLAG-β2-ADR as indicated were fed with anti-FLAG antibody and treated with isoproterenol (20 min, 10 μM). Flow cytometry was used to measure percentage of receptor internalized by loss of surface receptor fluorescence in cells expressing receptor with either pcDNA3 (control) or myc-Hrs. B, flow cytometry was used to measure percentage of receptor recycled by quantifying surface receptor fluorescence following agonist-induced endocytosis after 1 h of agonist washout in the presence of antagonist alprenolol (10 μM). C, extracts from HeLa cells transfected with siRNAs as indicated were subjected to Western blot and probed with antibodies to Hrs or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control). D, measurement of WT β2-ADR recycling by flow cytometry in cells transfected with either nonsense control or Hrs siRNAs. Data represent the means ± S.E. from three independent experiments. E, percentage of receptor recycling following agonist exposure was measured by quantitation of surface receptor fluorescence by flow cytometry in cells expressing WT or mutant β2-ADRs following control or Hrs siRNA transfection. Cells were treated as in A and B. Data represent the means ± S.E. from five independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with control.
sensitivity of the AKANKAA mutant was not as marked in \( \beta_2 \) ADRs where either the upstream acidic or dileucine residues were mutated independently (Fig. 4B, EKEN/AAK and LL/AA), suggesting that both are required for fully conferring Hrs-dependent recycling on the wild type \( \beta_2 \) ADR. Furthermore, mutating the downstream acidic residues together with the dileucine (LLCED/AACAA mutant receptor) did not reduce Hrs sensitivity nearly as much as mutating the dileucine together with upstream acidic residues (AKANKAA), and this resulted in sensitivity to myc-Hrs overexpression that was closely similar to mutating the dileucine (LL/AA mutant) by itself (Fig. 4B). These results provide additional support for a specific role of the EKENKLL sequence in targeting \( \beta_2 \) ADRs for Hrs-dependent recycling.

We next examined if the EKENKLL sequence is required to sort \( \beta_2 \) ADRs to the endogenous Hrs-dependent recycling mechanism by knocking down expression of cellular Hrs using a previously characterized siRNA. This approach has been previously shown to produce an effective depletion of endogenous Hrs from HeLa cells and effectively inhibits Hrs-dependent trafficking processes (16, 22, 23). Knockdown was confirmed by immunoblotting of cell extracts using an antibody recognizing endogenous Hrs (Fig. 4C). Plasma membrane recycling of WT \( \beta_2 \) ADRs was strongly inhibited by siRNA-mediated knockdown of Hrs but not of nonsense control siRNA (Fig. 4D), as shown previously (16). Recycling was also significantly inhibited by a second RNA oligonucleotide duplex, which targets a distinct sequence in Hrs and effectively depleted endogenous Hrs (Fig. 4, C and D), confirming the specificity of the RNA interference approach used.

Recycling of the WT \( \beta_2 \) ADR, as shown previously (16), was strongly inhibited by Hrs knockdown (Fig. 4E, 1st pair of bars). In marked contrast, recycling of \( \beta_2 \) ADR AKANKAA mutant receptor was completely insensitive to Hrs knockdown (Fig. 4E, 2nd pair of bars). Mutation of either the dileucine or acidic residues separately (\( \beta_2 \) ADR LL/AA or EKEN/AKA mutants; Fig. 4E, 3rd and 4th pairs, respectively) produced only a partial reduction in Hrs dependence, confirming an important role of both the dileucine and upstream acidic residues in this sequence. Mutation of the two downstream acidic residues (\( \beta_2 \) ADR LLCED/AACAA mutant) did not detectably reduce the Hrs dependence of recycling over the LL/AA mutant \( \beta_2 \) ADR (Fig. 4E, 5th pair of bars). This is fully consistent with the results observed under conditions of Hrs overexpression (Fig. 4B) and further confirms the specific requirement for the dileucine together with upstream acidic residues. Overall, the present results strongly support an important and specific function of the identified acidic dileucine sequence in sorting the wild type \( \beta_2 \) ADR from default to Hrs-dependent mechanisms.

**DISCUSSION**

Previous studies identified a specialized mechanism controlling plasma membrane recycling of the \( \beta_2 \) ADR (5–7, 15), which utilizes similar endocytic compartments as nonselective recycling via bulk membrane flow (26) but is distinguished mechanistically from default recycling by the following: 1) dependence on a “recycling sequence” comprised of a PDZ ligand present in the distal \( \beta_2 \) ADR C-tail, and 2) specific requirement for the endosome-associated sorting protein Hrs. A fundamental problem in understanding this mechanism has been the observation that disruption of either the distal recycling signal or depletion of Hrs does not result in efficient recycling of receptors by default. This raised the question of whether there exists additional structural information in the \( \beta_2 \) ADR that targets internalized receptors to the specialized (Hrs-dependent) recycling mechanism and effectively prevents default recycling of receptors. The present results show that this is indeed true and define a specific sequence in the \( \beta_2 \) ADR cytoplasmic tail that mediates this sorting. Thus the defined sequence functions, in essence, to “switch” the endocytic trafficking of receptors from an Hrs-independent, default-type mechanism to the Hrs/PDZ-dependent mechanism mediating recycling of the wild type \( \beta_2 \) ADR. To our knowledge this “mode-switching” function is unique among endocytic sorting signals defined previously in GPCRs (Fig. 5). Mutation of the novel sorting sequence produced a dramatically different phenotype (default recycling) than mutation of a previously defined recycling signal present in the distal \( \beta_2 \) ADR C-tail, which greatly inhibits recycling when mutated (5–7,15). This suggests an epistatic relationship between these distinct sorting sequences, in which the acidic dileucine sequence functions upstream of both Hrs- and the PDZ-dependent recycling sequence, mediating the initial sorting of endocytosed receptors to the specialized recycling mechanism (Fig. 5). This hypothesis accounts for the ability of receptors with a mutated EKENKLL to effectively bypass both Hrs and PDZ requirements and, in turn, also explains why the wild type \( \beta_2 \) ADR, in contrast to a number of other integral membrane proteins, recycles inefficiently by default.

The sorting sequence identified in the \( \beta_2 \) ADR tail resembles a group of acidic cluster dileucine motifs that mediate a variety of endocytic sorting functions for other membrane cargo, including endosome-to-lysosome and trans-Golgi network-to-endosome trafficking (25). In particular, this \( \beta_2 \) ADR sequence
was initially targeted for its similarity to those acidic-cluster dileucine sequences within the mannose 6-phosphate receptors. For these receptors this sequence is required for trafficking by binding specifically to the VHS (Vps27p, Hrs, STAM) domain of the GGA (Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding protein) (25). Moreover, our previous data have suggested a role for the VHS domain in Hrs in recycling of the β2ADR (16). Therefore, identification of a similar sequence in the β2ADR tail region that confers Hrs dependence was of extreme interest, particularly because a function of this type of sorting motif in a GPCR, and in endosome-to-plasma membrane trafficking of any membrane protein, has not been described previously.

Our results suggest a role for both the dileucine and acidic residues in the EKENKLL sequence in Hrs-dependent targeting, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on receptor recycling. These residues in this novel targeting sequence seem to have a specific function because alanine replacement of the dileucine combined with downstream sequence seem to have a specific function because alanine alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses in Hrs and PDZ dependence on internalization, because mutating either the acidic or dileucine residues alone had intermediate losses. This sequence resembles other acidic dileucine motifs but as discussed above, its sorting function is distinct from those previously described in other membrane proteins. In general, acidic dileucine sequences are thought to function biochemically by linking membrane cargo to various cytoplasmic adaptor proteins (25). One potential binding partner for the novel β2ADR sorting sequence is Hrs itself. We think this is unlikely, however, because depletion of Hrs and disruption of the acidic dileucine sequence produce fundamentally different trafficking phenotypes (endosomal retention versus default trafficking) (16). Also, we have so far not been able to establish a direct biochemical interaction between the novel sorting sequence and Hrs. Thus we presently favor the hypothesis that this sequence functions by binding to a distinct and, as yet, unidentified adaptor protein.

Another goal for future study is to understand, at the level of individual endosomal compartments and their membrane dynamics, how the acidic dileucine sequence mediates its recycling mode-switching function. The model depicted in Fig. 5 suggests that the EKENKLL sequence functions to sort receptors from early endosomes to a distinct population of recycling endosomes mediating Hrs-dependent, but not default, trafficking to the plasma membrane. This model is consistent with a recent study identifying an Hrs-containing protein complex, associated with a subset of endosomes, which mediates a rapid component of plasma membrane recycling (27). It is also possible that default and Hrs-dependent recycling occur via a shared population of endosomal intermediates. In this case, the EKENKLL sequence could function to deliver or link internalized receptors to a specific region or domain of the early endosome membrane that is associated with critical endocytic trafficking proteins such as Hrs. This possibility is consistent with the evidence that Hrs-containing protein complex(es) are organized in distinct microdomains of the early endosome membrane (17, 18, 22, 28).

Because efficient recycling can occur without a requirement for any known sorting signals (as for transferrin receptor and V2R 362T), why does such complexity exist in the recycling of the β2ADR? One possibility is to provide flexibility of receptor regulation. Rapid recycling of the β2ADR, although advantageous for functional recovery of signaling under some conditions (e.g. after short term agonist activation), could be deleterious under other conditions (such as prolonged or excessive signaling). It is therefore conceivable that β2ADR endocytic sorting can be flexibly reprogrammed under different physiological conditions. The present results provide initial insight to how pronounced alterations in the endocytic trafficking itinerary of a prototypic signaling receptor can be achieved.

In conclusion, the present results make a significant step forward in elucidating the mechanistic basis for regulated endocytic trafficking of GPCRs by identifying a novel type of acidic dileucine sequence in the β2ADR that is critical for sorting internalized receptors to the specialized, Hrs-dependent recycling mechanism. The mode-switching property of this sequence, which is unique among presently identified endocytic sorting sequences, also suggests a basis for flexible regulation of receptor trafficking fates.

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