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Mutating the dileucine motif of the human β_2 -adrenoceptor reduces the high initial rate of receptor phosphorylation by GRK without affecting postendocytic sorting

Hibah O. Awwad^{a,1}, Ellen E. Millman^b, Estrella Alpizar-Foster^a, Robert H. Moore^b, and Brian J. Knoll^{a,*}

^aDepartment of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX 77204, United States

^bDepartment of Pediatrics, Baylor College of Medicine, 6621 Fannin, CCC1040, Houston, TX 77030, United States

Abstract

The internalization of β_2 -adrenoceptors after agonist activation results in a desensitized and phosphorylated receptor that either resensitizes by recycling to the cell surface or becomes degraded by postendocytic sorting to lysosomes. The duration and physiological effects of agonists therefore depend on β_2 -adrenoceptor sorting, highlighting the importance of sorting signals. Dileucine motifs within other membrane proteins act as signals for endocytosis and/or postendocytic sorting, and the β_2 -adrenoceptor has a dileucine motif within helix 8 that might play a role in efficient receptor recycling and/or downregulation. β_2 -adrenoceptor internalization and sorting were studied in HEK293 cells stably expressing wild type or mutant dialanine L339A,L340A β_2 -adrenoceptors. The mutant β_2 -adrenoceptors showed a significantly lower initial rate of phosphorylation at the prominent G-protein coupled receptor kinase (GRK) sites Ser355 and 356 compared to wild type β_2 -adrenoceptors. Furthermore, the agonist-induced endocytic rate constant for L339A,L340A β_2 -adrenoceptors was reduced to ~25% that of wild type β_2 -adrenoceptors, which resulted in a similar reduction in agonist-induced downregulation. Internalized L339A,L340A β_2 -adrenoceptors recycled to the surface with a rate and extent similar to that of wild type β_2 -adrenoceptors. Therefore, although the role of L339,L340 in β_2 -adrenoceptor recycling or postendocytic sorting seems minimal, we conclude that L339, L340 is required for the initial high rate of phosphorylation by G-protein coupled receptor kinases at Ser355,356, which in turn is required for efficient β_2 -adrenoceptors endocytosis.

Keywords

β_2 -adrenoceptor; Sorting motif; G-protein coupled receptor kinase; Phosphorylation; Recycling; Endocytosis

1. Introduction

The cellular sorting of signal-transducing receptors is often controlled by short cytoplasmic amino acid sequences called sorting motifs. These sequences bind adaptors or chaperones in

the cytoplasm to mediate the transport of receptors from one cellular location to another. Understanding G-protein coupled receptor sorting motifs is crucial because receptor sorting plays a role in regulating physiological responses to agonists. β_2 -adrenergic receptors (β_2 -adrenoceptors) desensitize rapidly after activation by agonists, initially through rapid phosphorylation by G-protein coupled receptor kinases (GRKs) (Benovic et al., 1986) and protein kinase A (Clark et al., 1988). The phosphorylation of serines 355, 356 and 364 is critical for receptor endocytosis (Seibold et al., 2000; Vaughan et al., 2006). These phosphoserines act as docking sites for the scaffolding protein β -arrestin (Vaughan et al., 2006), which in turn triggers other signaling cascades (DeWire et al., 2007) and also binds adaptor protein 2 (AP-2) and clathrin, both of which are required for agonist-induced endocytosis of the β_2 -adrenoceptors into sorting endosomes (Goodman et al., 1996; Laporte et al., 1999). From this compartment, most β_2 -adrenoceptors return rapidly to the cell surface and a small fraction sorts to lysosomes for degradation (Moore et al., 2004; Morrison et al., 1996; von Zastrow and Kobilka, 1992). During chronic agonist exposure, this fraction becomes significant and total receptor numbers decline; a process called down-regulation (Gagnon et al., 1998; Moore et al., 1999a,b).

A dileucine motif at residues 339 and 340 is necessary for efficient β_2 -adrenoceptor internalization but not for coupling to the G-protein G_s (Gabilondo et al., 1997). Dileucine motifs are known to mediate the internalization and/or lysosomal targeting of various other membrane proteins by binding to adaptors such as AP-2 and AP-3 (Boonen et al., 2008; Marks et al., 1996; Sandoval et al., 1994; Trowbridge et al., 1993). These motifs typically consist of adjacent leucines or one leucine with a neighboring isoleucine, methionine or valine (Bonifacino and Traub, 2003; Letourneur and Klausner, 1992; Marks et al., 1996; Sandoval et al., 2000). Dileucine-based motifs usually fall into two categories, [DE]xxxL[L/I] and DxxLL, (D/E: acidic residues, x: any amino acid, L: leucine, I: isoleucine) (Bonifacino and Traub, 2003; Sorkin, 2004). [DE]xxxL[LI] motifs are implicated in receptor internalization and lysosomal targeting, while DxxLL motifs appear to be involved mainly in transport between the *trans* Golgi-network (TGN) and endosomes (Bonifacino and Traub, 2003).

Although the β_2 -adrenoceptor's dileucine motif was initially identified because it is necessary for efficient receptor internalization, the role of this motif in postendocytic sorting is unknown. Also, the importance of the nearby serines 355 and 356 and their phosphorylated states relative to the dileucine function is unknown. We have addressed these questions in β_2 -adrenoceptor sorting using HEK293 stable cell lines expressing either wild type β_2 -adrenoceptors or β_2 -adrenoceptors with mutated dileucines to study receptor endocytosis, phosphorylation of serines 355 and 356, and postendocytic β_2 -adrenoceptor sorting.

2. Materials and methods

2.1. Reagents

Rabbit polyclonal antibodies to the β_2 -adrenoceptors carboxyl-terminus and the β_2 -adrenoceptors phosphoserines 355,356 (pSer355,356) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were from Jackson ImmunoResearch (West Grove, PA) and (–) isoproterenol bitartrate was from ICN Biochemicals (Irvine, CA). (–) Propranolol and poly-D-lysine (PDL) were from Sigma-Aldrich (St. Louis, MO), and digitonin from Calbiochem (San Diego, CA). Dulbecco's modified Eagle medium (DMEM) with low glucose, Dulbecco's phosphate-buffered saline (PBS), precast 10% Tris–glycine gels, fetal bovine serum (FBS), Geneticin (G418), and 100X penicillin/streptomycin were from Invitrogen (Carlsbad, CA). ^3H CGP-12177 (~100 Ci/mmol) was from Perkin Elmer Life

and Analytical Sciences (Boston, MA). FuGene6 transfection reagent was from Roche Applied Sciences (Indianapolis, IN).

2.2. Construction of the β_2 -adrenoceptor L339A,L340A mutant

A DNA fragment encoding a FLAG-tagged wild type β_2 -adrenoceptor (Kobilka, 1995) was subcloned into the vector pcDNA3.1(+) (Invitrogen). We then created the mutation L339A,L340A by using the QuikChangeTM mutagenesis kit (Stratagene, LaJolla, CA) with the FLAG-tagged β_2 -adrenoceptor plasmid as a template and the primer sequence GCCTTCCAGCAGGCTGCGTGCCTGCGCAGGTCTTCTTTGAAGGCC and its reverse. Nucleotide sequencing was performed to verify the sequences of the plasmids.

2.3. Generation of stable cell lines in HEK293 cells

Human embryonic kidney (HEK293) cells (American Type Culture Collection (Manassas, VA)) were cultured on 100 mm tissue culture plates in DMEM containing 10% FBS and 1X penicillin/streptomycin. Stable lines expressing either the wild type β_2 -adrenoceptors or L339A,L340A β_2 -adrenoceptors were created by transfecting plasmids into HEK293 cells with 11 μ g of DNA and 17 μ l of FuGene6 per plate. Forty-eight hours post-transfection, the cells were split into media containing 800 μ g/ml G418 to select for clones expressing the transfected plasmids. Colonies were transferred to separate wells and propagated with 400 μ g/ml of G418 for further screening. β_2 -adrenoceptor-expressing cell lines were identified using surface radioligand binding with [³H] CGP-12177 as explained in detail below. The K_D for binding of [³H] CGP-12177 to mutant and wild type receptors at 4 °C was not significantly different (Table 1).

2.4. Receptor internalization kinetics

Clones growing in 24-well clusters were treated with isoproterenol (10 μ M) for varying times up to 20 min, then washed with cold DMEM containing 10 mM HEPES, pH 7.4 (DMEM-H) followed by a 90 min incubation with 9 nM [³H] CGP-12177 in DMEM-H at 4 °C to selectively bind surface receptors. The cells were washed and lysed with 0.1% SDS/ 0.1% NP-40, and the lysates were then counted in a β -scintillation spectrometer. Non-specific binding was assessed by parallel incubations that included 3 μ M propranolol and was approximately <1%. The first-order rate constants for endocytosis (k_e) and recycling (k_r) were derived by nonlinear regression as described previously (Morrison et al., 1996).

2.5. Receptor recycling

HEK293 cells expressing L339A,L340A or wild type β_2 -adrenoceptors were treated with isoproterenol (5 μ M) for 20 min, and then washed with ice-cold DMEM-H, fed with fresh 37 °C medium and incubated for varying times up to 60 min. The cells were then chilled and incubated for 90 min with 9 nM [³H] CGP-12177 in DMEM-H at 4 °C to selectively bind surface receptors. Surface receptor levels in isoproterenol-treated cells were plotted as fractions of that in untreated cells.

2.6. Receptor downregulation

HEK293 cells expressing L339A,L340A or wild type β_2 -adrenoceptors were treated with varying concentrations of isoproterenol in an anti-oxidant solution (0.1 mM ascorbic acid and 1 mM thiourea) as the vehicle for 24 h at 37 °C or with anti-oxidant vehicle alone. The cells were then washed four times with cold DMEM-H and incubated at 4 °C for 90 min in DMEM-H with 9 nM [³H] CGP-12177 and 0.05% digitonin to bind total receptors. Digitonin permeabilizes cellular membranes, enabling the hydrophilic antagonist [³H] CGP-12177 to bind total cellular receptors (Moore et al., 1999a,b). Receptor levels in

isoproterenol-treated cells were calculated as a fraction of receptors in cells treated with anti-oxidant vehicle alone for the same time interval.

2.7. Immunofluorescence microscopy

Cells were treated with isoproterenol in the presence of 100 μ M leupeptin (a protease inhibitor) to allow receptors to accumulate in lysosomes without being degraded. The cells were washed with phosphate-buffered saline with 1.2% sucrose and fixed with 4% paraformaldehyde at room temperature, then incubated for 20 min with 0.34% L-lysine/0.05% Na-periodate to quench free aldehydes that could contribute to an increased background fluorescence signal. Following a wash, the cells were incubated with 0.2% Triton X-100 for 10 min to permeabilize cell membranes and washed again. After blocking with 10% normal goat serum, cells were incubated in primary antibodies for 1 h at room temperature, then washed and incubated in secondary antibodies for 30 min before being washed and mounted as explained below. Primary and secondary antibodies were prepared in 0.2% normal goat serum/0.05% Triton X-100 at these dilutions: anticarboxy terminus β_2 -adrenoceptor (1:200), monoclonal mouse anti-LAMP-1 (1:200) (LAMP-1; lysosomal associated membrane protein-1), Texas Red goat anti-rabbit (1:400) or Alexa 594 goat anti-rabbit (1:500), and Alexa 488 goat anti-mouse (1:400). The coverslips were mounted in Mowiol containing 2.5% DABCO (an anti-fade reagent, 1,4-diazabicyclo [2.2.2]octane), and left to harden overnight. Images were captured at 1000 $\times$ magnification using the IX70-model inverted Olympus microscope with a Quantix[®] back-thinned CCD camera. Thirty-two optical sections (z-stacks) of 200 nm were obtained and deconvolved using the constrained iterative algorithm of Hiraoka et al. (1987) based on the obtained point spread function (PSF) with Isee software (Raleigh, NC). After deconvolution, the image contrast was adjusted in Adobe Photoshop 5.5.

2.8. Phosphorylation of β_2 -adrenoceptor at Ser355,356

The phosphorylation of β_2 -adrenoceptors was measured using an antibody that binds phosphoserines 355 and 356 on immunoblots as described previously (Iyer et al., 2006; Tran et al., 2004). HEK293 cells expressing L339A,L340A or wild type β_2 -adrenoceptors were treated with 10 μ M isoproterenol for up to 20 min (for time courses) or with varying concentrations of isoproterenol for 2 min (1 nM, 10 nM, 100 nM, 300 nM, 1 μ M, 10 μ M and 100 μ M) for the dose-response measurement. After washing with PBS, the cells were lysed with phosphorylation buffer (10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 0.2 mM NaVO_3 , 0.1 μ M okadaic acid, Complete[®] mini protease inhibitor). Laemmli's sample buffer was then added and the samples were heated at 60 $^{\circ}\text{C}$ for 15 min. Ten micrograms of total protein were deglycosylated with protein N-glycosylase F then electrophoresed and blotted to membranes. The membranes were first probed with anti-phosphoserines 355,356, then stripped and probed with anti- β_2 -adrenoceptors carboxyl-terminus antibody. For each antibody, antibody-antigen complexes were detected by binding to goat anti-rabbit-HRP and detected using the Westpico SuperSignal chemiluminescence kit (Pierce, Milwaukee, WI, U.S.A.). Imaging of the blots was performed using a FluorChem 8800 CCD imaging device (Alpha Innotech, San Leandro, CA). Signals were measured within the linear range of the instrument's level of detection as determined by blotting serial dilutions of receptor-containing extracts. The densities of the bands given by phospho- β_2 -adrenoceptors were normalized to the corresponding total receptor bands. Receptor phosphorylation was calculated as a fraction of the maximum phosphorylation of wild type receptors after 5 min of agonist exposure (for the time course) or as a percentage of the maximum phosphorylation for wild type receptors at 10 μ M isoproterenol (for the dose-response curve).

2.9. Data analysis

The data were analyzed by curve fitting using the program GraphPad Prism v. 4.0c (GraphPad Software, LaJolla, CA). Statistical analyses used are indicated in the figure legends.

3. Results

HEK293 cell lines were stably transfected to express either wild type or mutant L339A,L340A β_2 -adrenoceptors and clones were characterized as shown in Table 1. All experiments were performed in two separate cell lines for both wild type and mutant β_2 -adrenoceptors expressing similar levels of β_2 -adrenoceptors (1–1.9 pmol/mg) to minimize differences caused by clonal variation.

Although agonist-induced internalization of L339A,L340A β_2 -adrenoceptors (Fig. 1A) showed a significant reduction compared to wild type β_2 -adrenoceptors, functionally, the mutant β_2 -adrenoceptors activity on adenylate cyclase was similar to that of wild type β_2 -adrenoceptors (Gabilondo et al., 1997, data not shown). To understand the impact of the dileucine mutation on internalization kinetics (a function of both endocytic and recycling rate constants), cells were treated with 10 μ M isoproterenol (a strong β -agonist) for the indicated time points up to 20 min, then surface receptors were selectively labeled with the hydrophilic antagonist [3 H]CGP12177 as described in Materials and methods. The internalization time course curves were modeled by nonlinear regression to derive the first-order rate constants for endocytosis, as described by us earlier (Morrison et al., 1996). For both cell lines expressing L339A,L340A β_2 -adrenoceptors, the endocytic rate constants were reduced to 25–30% that of wild type β_2 -adrenoceptors (Fig. 1A, Table 2). In a separate analysis of the first 3 min of internalization (during which recycling and other postendocytic sorting events are not significant), data were transformed and modeled by linear regression. The endocytic rate constants so derived were reduced in L339A,L340A β_2 -adrenoceptors to 25–30% of that of the wild type β_2 -adrenoceptors (Fig. 1B, Table 2), similar to the results obtained by nonlinear regression. Concentration–response curves were performed to determine the isoproterenol concentrations needed to produce steady-state receptor internalization (i.e. 20 min of exposure). The half-maximal effective concentrations (EC_{50} s) for internalization of the dileucine mutated receptors were increased approximately six-fold compared with wild type receptors (Fig. 2 and Table 3).

The rate and extent of β_2 -adrenoceptor internalization is a function of both receptor endocytosis and recycling (Morrison et al., 1996). In principle, an increase in the rate of recycling could contribute to a decrease in receptor internalization. As shown in Table 2, the recycling rate constants of L339A,L340A β_2 -adrenoceptors derived from Fig. 1A were not greater than the rate constants of the wild type β_2 -adrenoceptors. To verify these derived recycling rates, we assessed the recycling of L339A,L340A β_2 -adrenoceptors directly. Cells were treated with isoproterenol for 20 min to internalize β_2 -adrenoceptors, then washed and incubated at 37 °C in the absence of isoproterenol during β_2 -adrenoceptor recycling to the cell surface. Surface receptor levels were measured by binding with [3 H] CGP12177 at various time points after agonist removal. Fewer L339A,L340A β_2 -adrenoceptors were internalized after 20 min of isoproterenol treatment as expected, however, those receptors that internalized returned to the cell surface at a rate similar to that of wild type β_2 -adrenoceptors (Fig. 3).

Next, we determined the role of the dileucine motif in the sorting of β_2 -adrenoceptors to the degradative pathway. Internalization and downregulation were measured in parallel experiments after 24 h of treatment with isoproterenol at varying concentrations (Fig. 4A). β_2 -adrenoceptor downregulation is dependent on the levels of internalized β_2 -adrenoceptors,

and since L339A,L340A β_2 -adrenoceptors internalize to a lesser extent than wild type receptors, it was essential that we compare downregulation when levels of both wild type and mutant β_2 -adrenoceptors internalized to the same extent ~40–45% (3 nM and 1 μ M isoproterenol respectively). At these respective concentrations of isoproterenol, the corresponding downregulation of both L339A,L340A β_2 -adrenoceptors and wild type β_2 -adrenoceptors was ~15–20%. This result suggests that the net downregulation defect shown by the L339A,L340A β_2 -adrenoceptors after 24 h of isoproterenol is a sequential response to the decrease in receptor endocytosis. This is further supported by immunofluorescence microscopy, where L339A,L340A β_2 -adrenoceptors show only limited localization with the lysosomal marker (LAMP-1) after a 24 h treatment with 10 μ M isoproterenol (Fig. 4B).

Altogether, these results confirm that the dileucine motif is much more important for receptor endocytosis than for postendocytic sorting. We next asked whether the rate of phosphorylation at β_2 -adrenoceptor serines 355 and 356 by GRK, a determining factor in the rate of receptor endocytosis (January et al., 1997), was affected by the dileucine mutation. At a saturating concentration of agonist, the phosphorylation time course of L339A,L340A β_2 -adrenoceptors at GRK sites is significantly different from wild type β_2 -adrenoceptors (Fig. 5). Wild type β_2 -adrenoceptor phosphorylation peaks within 2 min of isoproterenol treatment and is maintained for 20 min, whereas the rapid initial phase of GRK phosphorylation is lost in L339A,L340A β_2 -adrenoceptors, with a delayed peak in phosphorylation. However, the extent of phosphorylation of both wild type and L339A,L340A β_2 -adrenoceptors reached similar maximal levels within 20 min of isoproterenol treatment (Fig. 5). This temporal pattern of β_2 -adrenoceptor phosphorylation has been observed previously using partial agonists, and in those cases, the EC_{50} s for maximal phosphorylation at serines 355 and 356 were increased. To determine the EC_{50} for maximal phosphorylation of serines 355 and 356 we chose the 2 min time point for measurements. The EC_{50} of β_2 -adrenoceptor phosphorylation was increased by 10–20-fold in L339A,L340A β_2 -adrenoceptors compared with wild type β_2 -adrenoceptors (Fig. 6, Table 3). The phosphorylation of serines 355 and 356 in L339A,L340A β_2 -adrenoceptors after 2 min of isoproterenol exposure reached only 35% of the wild type even at 100 μ M isoproterenol (Fig. 6), suggesting that the delayed phosphorylation defect seen at serines 355 and 356 of the L339A, L340A β_2 -adrenoceptors cannot be fully rescued by increasing receptor occupancy by agonists.

4. Discussion

The dileucine motif of the β_2 -adrenoceptors at position 339,340 is highly conserved in G-protein coupled receptors (Huynh et al., 2009) and its neighboring residues resemble those required for the internalization and postendocytic sorting of a number of transmembrane proteins. Our study directly addressed the role of the β_2 -adrenoceptor's dileucine motif in postendocytic sorting and receptor phosphorylation. Mutating the dileucine motif in the β_2 -adrenoceptors does not have a significant impact on β_2 -adrenoceptor coupling (Gabilondo et al., 1997), and we have shown that β_2 -adrenoceptor recycling and downregulation are relatively unaffected. However, mutating the dileucine motif in the β_2 -adrenoceptors significantly reduced the initial rapid rate of β_2 -adrenoceptor phosphorylation by GRK, which is required for rapid receptor endocytosis.

Our findings highlight the importance of the sequence of protein or ligand interactions involved in β_2 -adrenoceptors phosphorylation and internalization. In other membrane proteins, dileucine motifs commonly function as internalization motifs by interacting with the heterotetrameric adaptor protein AP-2. Recent structural studies have shown that dileucine motifs compete with the β_2 subunit of AP-2 to bind to the σ_2 subunit of AP-2 (Kelly et al., 2008). However, if this were the mechanism for the dileucine-mediated

internalization of β_2 -adrenoceptors, one would not expect to see a reduction in phosphorylation by GRK in the mutated β_2 -adrenoceptors, since phosphorylation should occur prior to AP-2 interactions. In a previous study (Iyer et al., 2006), we showed that inhibiting endocytosis with dominant-negative dynamin expression had no effect on the rate of phosphorylation by GRK. Thus, the primary effect of the dileucine mutation seems to be on GRK phosphorylation and not on mechanisms further downstream. A reduction in phosphorylation at the GRK sites would lead to reduced recruitment of β -arrestin and AP-2, and reduced clathrin-mediated endocytosis. AP-2 knockdown using siRNA further inhibited L339A,L340A β_2 -adrenoceptors internalization (unpublished data), suggesting that the β_2 -adrenoceptor dileucine motif does not function primarily through direct AP-2 interactions like other dileucine motifs. On the contrary, with a reduced rate and extent of internalization, L339A,L340A β_2 -adrenoceptors should be easily accessible to GRK phosphorylation at the plasma membrane. Also, L339A, L340A β_2 -adrenoceptors reach similar levels of phosphorylation within 20 min of adding isoproterenol, and although the phosphorylation of the mutant receptor is significantly delayed compared to wild type, this indicates that the L339A,L340A β_2 -adrenoceptors are capable of being phosphorylated by GRK to levels similar to wild type β_2 -adrenoceptors.

The mutation of the dileucine motif to alanines does not seem to change the helical structure of helix 8 in β_2 -adrenoceptors (unpublished computer-modeling) and it also maintains the amphipathic nature required for its function (Huynh et al., 2009). Therefore, it is unlikely that a conformational change in helix 8 explains the reduced rate of phosphorylation by GRK of L339A,L340A β_2 -adrenoceptors. However, recent studies on the C5a receptor show that although a mutated dileucine in helix 8 did not affect direct binding of C5a receptors with GRKs *in vitro* (Suvorova et al., 2008), trypsin proteolysis revealed some conformational changes in the mutant C5a receptor. Therefore, we cannot rule out that the L339A,L340A mutation might cause a significant conformational change in β_2 -adrenoceptors. Our data support the concept that a decreased *initial rate* of β_2 -adrenoceptors phosphorylation by GRK, rather than the total phosphorylation during prolonged agonist exposure, causes the reduced rate of L339A,L340A β_2 -adrenoceptors endocytosis. A similar correlation was previously shown when receptor phosphorylation was varied by using a range of partial β -agonists. Partial agonists caused a greatly reduced initial rate of β_2 -adrenoceptors phosphorylation, however, after prolonged exposure, phosphorylation induced by partial agonists reached an extent similar to that induced by strong agonists (January et al., 1997). Similarly, L339A,L340A β_2 -adrenoceptors eventually were phosphorylated as much as wild type β_2 -adrenoceptors after prolonged exposure to a strong agonist (Fig. 5). This could be due to persistent presence of receptors on the cell surface (due to reduced endocytosis), where they would be continuously available as substrates to GRK. Our data may be interpreted such that the L339A,L340A β_2 -adrenoceptors exist in a conformation that requires prolonged activation by agonist at high occupancy levels (Figs. 5 and 6) before wild type levels of phosphorylation are achieved.

Though we can assay the phosphorylation only of serines 355 and 356, another serine residue at 364 is also an important site of phosphorylation by GRK. Thus we might expect phosphorylation of serine 364 to be reduced by the L339A,L340A mutation. However, the magnitude of the effect of the L339A,L340A mutation on endocytosis and phosphorylation is somewhat less than the effect of combined mutations at GRK sites serines 355, 356 and 364. The endocytic rate of the triple mutant is ~12% of the wild type β_2 -adrenoceptor (Vaughan et al., 2006), while that of the L339A,L340A mutant is 24–30% that of the wild type β_2 -adrenoceptor. The triple serine mutant receptor is phosphorylated to only 5% that of the wild type β_2 -adrenoceptor after 2 min of strong agonist exposure (Seibold et al., 2000), while the L339A,L340A mutant is phosphorylated to 30–40% of the wild type β_2 -adrenoceptor during that time interval. These comparisons are consistent with the idea that

the L399A,L340A mutation has indirect effects on the phosphorylation of serines 355 and 356 by way of allosteric mechanisms that are less severe than is obtained by direct removal of the GRK substrates themselves (as in the triple serine mutant). Structural experiments on helix 8 of the β_2 -adrenoceptors would be the best way to understand these observations, especially since the dileucine motif is located between the NPxxY(x)5,6F motif (responsible for G-protein coupling and internalization), and the palmitoylation sites at cysteine 341. However, we can conclude that the dileucine motif of the β_2 -adrenoceptor seems to play an important role in fine-tuning β_2 -adrenoceptor internalization by controlling the initial rate of β_2 -adrenoceptors phosphorylation by GRK at serines 355 and 356, without affecting its ability to couple to G-proteins.

The sequence in helix 8 near the dileucine motif has recently been referred to as the F(X)6LL motif, and has been shown to function as an export signal from the endoplasmic reticulum (Duvernay et al., 2009). Although L339A,L340A β_2 -adrenoceptor in our cell lines contained some intracellular receptors (as shown by immunofluorescence) which could be explained by reduced export from the endoplasmic reticulum, the mutant cell lines displayed the same surface expression levels as the wild type β_2 -adrenoceptor cell lines (Table 1). Therefore, in our cell lines and in the studies we have determined using the hydrophilic ligand, we measured L339A,L340A β_2 -adrenoceptors that reached the cell surface and it is apparent that export trafficking from the endoplasmic reticulum had no impact on any of our results.

Emerging evidence highlights the importance of understanding the structure of helix 8 and the conformational changes that occur to G-protein coupled receptors at every stage of activation and deactivation. Further structural analyses will help us clarify the importance of each amino acid in helix 8 and each step of the desensitization pathway in β_2 -adrenoceptors and other G-protein coupled receptors.

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Fig. 1.

The first-order rate constants for agonist-induced endocytosis are decreased in the L339A,L340A β_2 -adrenoceptors. Two independent HEK293 cell lines stably expressing wild type β_2 -adrenoceptors and two lines expressing L339A,L340A β_2 -adrenoceptors were constructed as described in the Materials and methods. Cells were grown in 24-well clusters, treated for varying times with 10 μ M isoproterenol, then chilled and incubated with 9 nM [3 H] CGP12177 to detect surface receptor levels expressed as a fraction of surface receptors in untreated cells as described in the Materials and methods. (A). Receptors remaining on the surface were plotted as a function of time after agonist addition. The internalization curves were modeled using nonlinear regression to obtain first-order rate constants for

endocytosis (k_e) and recycling (k_r) as described in Morrison et al. (1996). (B). The data points from the first 3 min of internalization were transformed and a linear regression was performed. The slopes of these lines approximate the endocytic rate constants. All rate values are shown in Table 2. * represents all points plotted on the graph that are significantly different between the wild type and L339A,L340A β_2 -adrenoceptor cell lines (unpaired Student's t -test, $P < 0.0001$). $N = 9-13$, data are presented as mean (symbol) $\pm$ S.E.M. (vertical bars). Some error bars are smaller than the symbols.

Fig. 2.

β_2 -adrenoceptors with the L339A,L340A mutation show an increase in the EC_{50} for internalization with isoproterenol. Stably transfected cells expressing wild type or L339A,L340A β_2 -adrenoceptors were treated with varying concentrations of isoproterenol for 20 min before being chilled and assayed for surface receptors by [3H] CGP12177 binding and receptor internalization calculated as fraction internalized after normalizing counts to those of surface receptor in untreated cells as described in Materials and methods. The EC_{50} values are shown in Table 3. Data are presented as mean $\pm$ S.E.M. * represents points plotted on the graph that are significantly different between the wild type and L339A,L340A β_2 -adrenoceptor cell lines (unpaired Student's *t*-test, $P < 0.05$). $N = 4-5$, and the data were curve-fitted to a sigmoidal dose-response curve using nonlinear regression analysis.

Fig. 3.

β_2 -adrenoceptor recycling is not significantly affected by the L339A,L340A mutation. Stably transfected cell lines expressing wild type receptors or L339A,L340A mutant receptors were treated with 5 μ M isoproterenol for 20 min, then washed at 4 °C to remove agonist before returning to 37 °C for various times up to 60 min. The return of receptors to the cell surface was assessed by binding to the hydrophilic radioligand [3 H]CGP12177 and the fraction of surface receptors was calculated as fraction of surface receptors in untreated cells as described in Materials and methods. The other pair of cell lines produced similar results. * represents points plotted on the graph that are significantly different between the wild type and L339A,L340A β_2 -adrenoceptor cell lines (unpaired Student's *t*-test, $P < 0.05$). $N = 5-6$, data are presented as mean $\pm$ S.E.M.

Fig. 4.

Downregulation of β_2 -adrenoceptors is not directly affected by the L339A,L340A mutation. (A). Stably transfected cells expressing wild type or L339A,L340A β_2 -adrenoceptors were treated with varying concentrations of isoproterenol for 24 h, then assessed for surface receptor levels using [3 H]CGP12177 binding, and for total receptor levels using the same radioligand, but in the presence of 0.05% digitonin, as described in Materials and methods. Data are presented as a percentage of untreated cells. $N = 5$, similar results were obtained with another pair of clones. Data are presented as mean (symbols) $\pm$ S.E.M (vertical bars). (B). HEK 293 stable cell lines expressing wild type (upper panel) and L339A,L340A mutant (lower panel) β_2 -adrenoceptors were grown on glass cover slips and treated with

isoproterenol (10 μ M) for 24 h or received no treatment in the presence of 100 μ M leupeptin. Receptors were immunolabeled using Texas Red secondary antibody and LAMP-1 was labeled green using FITC secondary antibody and images were deconvolved as described in Materials and methods. Arrows highlight yellow (overlap of red and green) punctate labeling where receptors localize with LAMP-1 in lysosomes. Images are representative of 5–10 cells from 4–5 different experiments in both pairs of cell lines.

Fig. 5. Receptors with the L339A,L340A mutations show a reduced rate of agonist-induced phosphorylation of serines 355,356. Stably transfected HEK293 cells expressing wild type or L339A,L340A β_2 -adrenoceptors were treated with 10 μ M isoproterenol for the indicated times, then chilled and harvested to assess receptor phosphorylation by immunoblotting, as described in the Materials and methods. (A) Quantification of immunoblots: phosphoserine band density values were normalized per receptor band density values. Phosphorylation was calculated as a fraction of maximum phosphorylation of wild type receptors at 5 min. Data are presented as mean $\pm$ S.E.M., $N = 4$. * represents significantly different from wild type receptor cell lines (unpaired t -test, $P < 0.05$). (B) A representative immunoblot of the

phosphorylation time course quantified in panel A, using antibodies against the carboxyl terminus of β_2 -adrenoceptor and its phosphoserines 355,356. Top panels, wild type β_2 -adrenoceptors; bottom panels, L339A. L340A β_2 -adrenoceptors.

Fig. 6.

Receptors with the L339A,L340A mutations show an increased EC_{50} for phosphorylation of serines 355,356. Stably transfected HEK293 cells expressing wild type or L339A,L340A β_2 -adrenoceptors were treated with isoproterenol for 2 min, then chilled and harvested for immunoblotting to detect phosphorylation of serines 355,356 as described in the Materials and methods. (A) Quantification of immunoblots: phosphoserine density values were normalized per receptor density values of the same lane. Phosphorylation was calculated as a percentage of maximum phosphorylation for wild type receptors at 10 μM of agonist. The data were fitted to a sigmoidal dose-response curve using nonlinear regression analysis. $N = 3-5$, Data are presented as mean (symbols) $\pm$ S.E.M (vertical bars). The EC_{50} values are

shown in Table 3. (B) A representative immunoblot of the phosphorylation dose–response using antibodies against the carboxyl terminus of β_2 -adrenoceptors and its phosphoserines 355,356. Top panels, wild type β_2 -adrenoceptors; bottom panels, L339A. L340A β_2 -adrenoceptors.

Table 1 β_2 -adrenoceptor expression by stable cell lines.

	Clone	K_D (nM)	B_{max} (p mol/mg)
Wild type	WT-3D	1.05 ± 0.18	1.96 ± 0.10
	WT-10	1.03 ± 0.18	1.83 ± 0.09
Mutant	LLAA-2	1.93 ± 0.23	1.66 ± 0.06
	LLAA-12	1.15 ± 0.20	1.70 ± 0.08

Data presented as values indicated $\pm$ S.E.M. K_D and B_{max} values determined by saturation binding of [3H] CGP-12177. $N = 3$ for all groups.

Table 2

Trafficking rate constants for wild type and mutant β_2 -adrenoceptors.

		First-order rate constants (min^{-1})		<i>N</i>
	Clone	k_e	k_r	
Wild type	WT-3D	0.263 ± 0.02	0.135 ± 0.016^a	5
	WT-10	0.268 ± 0.01	0.091 ± 0.011	9
Mutant	LLAA-2	0.068 ± 0.01^b	0.077 ± 0.016	4
	LLAA-12	0.079 ± 0.01^b	0.078 ± 0.016	13

Data presented as values indicated $\pm$ S.E.M. $N = 3-5$. Values were derived from regression analyses as illustrated in Fig. 1.

^a $P < 0.05$, significantly different from all others, Tukey's multiple comparison test.

^b $P < 0.001$, significantly different from both WT-10 and WT-3D.

Table 3

EC₅₀ for β_2 -adrenoceptor internalization and Ser355,356 phosphorylation.

		Internalization	Ser355,356 phosphorylation
Clone		Log EC ₅₀	Log EC ₅₀
Wild type	WT-10	-7.880 ± 0.095	-7.317 ± 0.207
	WT-3D	-7.594 ± 0.096	-7.702 ± 0.136
Mutant	LLAA-2	-7.109 ± 0.118	-6.702 ± 0.374
	LLAA-12	-7.168 ± 0.089	-6.702 ± 0.299

Data presented as values indicated ± S.E.M. $N = 3-5$. Values were derived from sigmoidal dose-response curve fitting as illustrated in Fig. 2 (internalization) and Fig. 6 (phosphorylation).