Agonist stimulation of the β2-adrenergic receptors (β2ARs) leads to their ubiquitination and lysosomal degradation. Inhibition of lysosomal proteases results in the stabilization and retention of internalized full-length β2ARs in the lysosomes, whereas inhibition of proteasomal proteases stabilizes newly synthesized β2ARs in nonlysosomal compartments. Additionally, a lysine-less β2AR (0K-β2AR) that is deficient in ubiquitination and degradation is not sorted to lysosomes unlike the WT β2AR, which is sorted to lysosomes. Thus, lysosomes are the primary sites for the degradation of agonist-activated, ubiquitinated β2ARs. To identify the specific site(s) of ubiquitination required for lysosomal sorting of the β2AR, four mutants, with lysines only in one intracellular loop, namely, loop 1, loop 2, loop 3, and carboxyl tail were generated. All of these receptor mutants coupled to G proteins, recruited β-arrestin2, and internalized just as the WT β2AR. However, only loop 3 and carboxyl tail β2ARs with lysines in the third intracellular loop or in the carboxyl tail were ubiquitinated and sorted for lysosomal degradation. As a complementary approach, we performed MS-based proteomic analyses to directly identify ubiquitination sites within the β2AR. We overexpressed and purified the β2AR from HEK-293 cells with or without prior agonist exposure and subjected trypsin-cleaved β2AR to LC-MS/MS analyses. We identified ubiquitinated lysines in the third intracellular loop (Lys-263 and Lys-270) and in the carboxyl tail (Lys-348, Lys-372, and Lys-375) of the β2AR. These findings introduce a new concept that two distinct domains in the β2AR are involved in ubiquitination and lysosomal degradation, contrary to the generalization that such regulatory mechanisms occur mainly at the carboxyl tails of GPCRs and other transmembrane receptors.

β2AR, a prototypical member of seven-transmembrane receptor superfamily plays critical roles in regulating a variety of physiological functions including maintenance of cardiovascular and pulmonary homeostasis (1–5) and has been a primary drug target for treating heart failure and asthma. β2AR couples to Gi and Gs; forms signaling complexes with β-arrestins 1 and 2, c-Src, and Cα1-1.2; and interacts with a variety of other proteins (6–15). Agonist-stimulated internalization and trafficking of the β2AR as well as its desensitization have been extensively characterized; however, novel regulatory mechanisms are still emerging.

Down-regulation and long term desensitization of the β2AR are regulated by receptor ubiquitination, which occurs upon agonist activation (16). The HECT domain E3 ubiquitin ligase Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) mediates β2AR ubiquitination and directs receptor trafficking to the lysosomal compartments (17). The adaptor protein β-arrestin2 plays a major role in this process, and its expression is required for receptor ubiquitination by Nedd4 (16, 17). Recent studies have also revealed novel roles of deubiquitinases, USP20 and USP33, which reverse β2AR ubiquitination, thus preventing lysosomal trafficking and promoting recycling as well as resensitization of the receptor (18). Accordingly, ubiquitin moieties on the receptor serve as signals that determine the intracellular itinerary and signaling status of agonist-activated β2ARs.

Ubiquitination of substrate proteins is canonically targeted at lysyl residues, whereby the ε amino group of a lysine in the substrate is covalently linked to the carboxyl group of the carboxyl-terminal glycine (glycine-76) of ubiquitin (19). The amino termini of substrate proteins are also targeted for ubiquitination or can serve as degradation signals as dictated by specific sequence motifs in the substrate, which determine protein half-life (20, 21). Although substrate ubiquitination is precisely regulated and involves sophisticated enzymatic machinery, it is not targeted at a unique sequence motif. On the other hand, ubiquitinated substrates are recognized by binding proteins that carry a highly conserved ubiquitin recognition motif (22).

By using receptor mutants in which some or all of the lysyl residues are replaced with arginines, ubiquitination of the β2AR and other seven-transmembrane receptors has been shown to regulate degradation of receptors; however, there are only a few studies identifying the target sites of receptor ubiquitination (23). For the chemokine receptor CXCR4, lysines within the carboxyl tail together with nearby phosphorylated serine residues function as a degradation motif (24). Mutation of the three carboxyl tail lysines abrogated CXCR4 ubiquitination and lysosomal sorting but did not affect receptor endocytosis. Mutation of a single lysine in vasopressin V2 receptor was shown to
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abrogate its ubiquitination and degradation (25). Recently, lysines in the carboxyl tail of the $\beta_2$AR were reported to be the main sites of receptor ubiquitination (26). However, mutation of these lysines did not eliminate receptor ubiquitination, and furthermore the roles of lysines in other domains of the $\beta_2$AR were not investigated.

Ubiquitination was originally discovered for its role in nonlysosomal degradation carried out by the multi-protease complex, 26 S proteasome (19). On the other hand, nontraditional roles of ubiquitin, especially in the endocytosis of yeast GPCRs and lysosomal degradation of membrane receptors, are being continuously reported (23, 27). Moreover, in many cases, both proteasomes and lysosomes are suggested to mediate degradation of seven-transmembrane receptors that are internalized to late endosomes, but the exact role of each degradative pathway is unclear (23).

Herein, we define the roles of proteasomes and lysosomes in the degradation of agonist-activated $\beta_2$ARs. Additionally, we have taken two complementary approaches, namely, characterizing mutant $\beta_2$ARs containing lysines in single intracellular domains as well as direct identification of $\beta_2$AR ubiquitination by mass spectrometry to determine the sites of ubiquitination within the $\beta_2$AR, which direct its lysosomal trafficking and long term desensitization.

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, and Antibodies—HEK-293 cells were obtained from ATCC and cultured in minimum essential medium supplemented with fetal bovine serum and penicillin and streptomycin. Mutant $\beta_2$ARs containing lysines only in one intracellular domain were generated by swapping domains between FLAG-$\beta_2$AR and FLAG-0K-$\beta_2$AR. FLAG-0K-$\beta_2$AR-mYFP was generated by cloning FLAG-0K-$\beta_2$AR- mYFP was generated by cloning FLAG-0K-$\beta_2$AR- mYFP plasmid vector. Antibodies to human $\beta_2$AR (H-20), LAMP2 (H4B4), and calreticulin (H10) were from Santa Cruz Biotech. FK1 ubiquitin antibody that is selective for polyubiquitin was purchased from Enzo Life Sciences. Unless specified otherwise, all other reagents were from Sigma.

Immunoprecipitation and Immunoblotting for Detecting Total $\beta_2$AR Levels—HEK-293 cells stably transfected with FLAG-$\beta_2$AR (2.5 pmol/mg cellular protein) were serum-deprived for 1 h and then stimulated with vehicle or 10 $\mu$M isoproterenol (Iso) for 24 h. To assess the role of proteasomes and lysosomes, the proteasomal inhibitor MG132 (10 $\mu$M) or lysosomal inhibitor leupeptin (50 $\mu$M) was added to samples along with Iso. When desired, cycloheximide (50 $\mu$g/ml) was added to the cells to inhibit protein synthesis. At the end of incubation, the cells were harvested in a lysis buffer containing 50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol. All of the buffers were supplemented with protease inhibitors. Harvested cells were further solubilized by adding n-dodecyl $\beta$-D-maltoside (final concentration, 1%) and incubating on a rotator at 4 °C for 2 h. After this, the samples were centrifuged, soluble extracts were prepared, and protein concentrations were determined by Bradford analysis. Equal amounts of lysates were mixed with anti-FLAG M2-agarose gel and rotated overnight at 4 °C. Nonspecific binding in the immunoprecipitate was eliminated by repeated washes with lysis buffer, and bound protein was eluted with sample buffer containing SDS. The samples were incubated at 37 °C for 30 min before SDS-PAGE. The eluted proteins were separated on a gradient gel (4–20%; Invitrogen) and transferred to nitrocellulose membrane for Western blotting. Anti-$\beta_2$AR antibody (H-20; Santa Cruz) was used at 1:3000 dilution to detect receptors in the immunoprecipitates. Chemiluminescence detection was performed using SuperSignal® West Pico reagent (Pierce). The signals were quantified by densitometry using Quantity One software (Bio-Rad).

Confocal Microscopy—HEK-293 cells were transiently transfected with indicated mutant $\beta_2$AR plasmids alone (see Fig. 4) or with $\beta$-arrestin2-GFP (see Fig. 3). 24 h post-transfection, the cells were plated on collagen-coated 35-mm glass bottom plates. 24 h later, the cells were starved for 1 h in serum-free medium and then stimulated with Iso for the desired times, fixed with 5% formaldehyde diluted in PBS containing calcium and magnesium, permeabilized with 0.1% Triton X-100™ in PBS containing 2% bovine serum albumin for 30 min, and incubated with appropriate primary antibody overnight at 4 °C, followed by the respective secondary antibody. Confocal images were obtained on a Zeiss LSM510 laser-scanning microscope using multitrack sequential excitation (488 and 568 nm) and emission (515–540 nm, GFP; 585–615 nm, Texas Red) filter sets.

cAMP Measurements—The cAMP biosensor ICUE2 (indicator of cAMP using Epac 2) was kindly provided by Dr. Jin Zhang (Johns Hopkins University). Cells stably expressing ICUE2 were transfected transiently with vector or desired receptor plasmids. After 24 h, the cells were plated on fibronectin-coated 96-well plates. Intracellular cAMP concentrations were measured as a FRET ratio as follows: cyan fluorescent protein intensity (438/32 emission band pass filters; Semrock) relative to FRET intensity (542/27 emission filter). The experiments were performed on a NOVOstar plate reader (BMG Labtech).

Radioligand Binding—Cell surface expression of WT and mutant $\beta_2$ARs were determined in parallel for cAMP experiments. A portion of the transfected cells were plated on poly-$\beta$-lysine-coated 24-well dishes (Biocoat) and labeled with 10 nM (−)[$^3$H]CGP-12177. Nonspecific binding was determined by displacement with 10 $\mu$M propranolol.

To measure the loss of total receptor by degradation, [125I](-)-iodocyanopindolol ([125I]CYP) radioligand binding on monolayers of cells on poly-$\beta$-lysine-coated 12-well dishes (Biocoat) was performed. The experimental cells were divided into two identical sets: one that was treated with agonist and the other treated with vehicle. Receptor levels in both sets were determined in parallel. Binding was performed in triplicate with 400 pm [125I]CYP in the presence or absence of the hydrophobic antagonist propranolol (10 $\mu$M, to define nonspecific binding). The samples were incubated at 37 °C for 1 h, after which the cells were placed on ice and washed several times with ice-cold PBS buffer containing calcium and magnesium. Finally, the cells were solubilized in 0.1 N NaOH and 0.1% SDS and counted for [125I]. The receptor number (total specific [125I]CYP binding sites) was determined after 24 h of Iso treatment and expressed as a percentage of receptor number assessed in nonstimulated cells.
Receptor Internalization—FLAG-tagged receptors expressed in HEK-293 cells in 12-well dishes were incubated with or without agonist for 30 min in serum-free medium at 37 °C. Cell surface receptors were labeled with M1 FLAG mAb and fluorescein isothiocyanate-conjugated goat antibody to mouse IgG as a secondary antibody. Receptor internalization was quantified as loss of cell surface receptors as measured by fluorescence-assisted cell sorting (flow cytometry facility, Duke University). Base-line cell fluorescence intensity was determined with washed unlabeled cells and cells incubated only with the fluorescein-labeled goat anti-mouse antibodies. The percentage of β2AR internalization was deduced as published previously (16, 28–32).

Human β2AR Purification—Human β2AR was purified from HEK-293 cells stably overexpressing wild type β2AR using alprenolol-Sepharose affinity resin (33). The β2AR stable cells were grown to confluence in minimum essential medium and starved overnight in serum-free medium. The cells were then treated without or with 10 μM ISO for 1 h prior to harvest. The harvested cells were lysed by three cycles of freeze and thaw in a hypotonic solution (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA), followed by mechanical disruption using a Dounce homogenizer. Crude membrane fractions were prepared from the above cell lysates by centrifugation at 14,000 rpm for 30 min in a SS34 rotor. The β2ARs were extracted from the crude membrane fractions by 1% n-dodecyl β-D-maltoside. Alprenolol-Sepharose affinity resin was then added to the extracted solution, and the mixture was incubated for 4 h on a 4 °C rotator. The receptor-bound alprenolol-Sepharose affinity resin was collected by centrifugation (1,000 × g for 1 min at 4 °C) and washed five times with 1 ml of ice-cold wash buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.1% n-dodecyl β-D-maltoside) and five times with 1 ml of ice-cold wash buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.01% n-dodecyl β-D-maltoside). The purified β2ARs were then eluted with 5 mM alprenolol in wash buffer B and stored at −100 °C before mass spectrometry analyses.

Online LC-MS/MS Analysis—The purified β2ARs were precipitated with methanol/chloroform (CHCl3) followed by resolubilization of the protein pellet using urea and in-solution digestion with trypsin. In brief, four volumes of methanol were added to receptor solution and mixed. One volume of chloroform was then added to the mixture. Phase separation was then accomplished by adding three volumes of water followed by vigorous mixing and centrifugation at 12,000 × g for 5 min. The upper aqueous phase was carefully aspirated, then four volumes of methanol were added to the organic phase to precipitate the receptor proteins. After centrifugation at 12,000 × g for 5 min, the supernatant was removed, and the samples were dried in a speed vacuum (Savant Instruments Inc., Farmingdale, NY). The receptor proteins were then resolubilized in a buffer (50 mM Tris-HCl, pH 8.0, 8 mM urea, 5 mM EDTA, and 0.005% n-dodecyl β-D-maltoside), reduced with 2 mM DTT (final concentration) at 65 °C for 30 min, and alkylated with 10 mM iodoacetamide at room temperature for 30 min in the dark. In-solution tryptic digestion was performed at 37 °C overnight in a solution (50 mM Tris-HCl, pH 8.0, 1 mM urea, 5 mM EDTA, and 0.005% n-dodecyl β-D-maltoside) with trypsin at a working concentration of 5 ng/μl. Digested peptides were purified by Stage-tip chromatography (34), lyophilized, and reconstituted in 2% (v/v) acetonitrile, 0.1% (v/v) TFA for LC-MS/MS analysis. LC-MS/MS experiments were performed on a Thermo Scientific LTQ-Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) equipped with a Finnigan Nanospray II electrospray ionization source (Thermo Electron), a Waters nanoACQUITY Ultra Performance LC™ system (Waters, Milford, MA). For the LC separation, tryptic peptides were injected onto a 75-μm × 150-mm BEH C18 column (particle size, 1.7 μm; Waters) and separated using a gradient of 5 to 40% acetonitrile with 0.1% formic acid, with a flow rate of 0.4 μl/min in 90 min. The LTQ-Orbitrap XL mass spectrometer was operated in the data-dependent mode using the FT10 strategy (35). In brief, for each cycle, one full MS scan (350–1800 m/z; acquired in the Orbitrap at 6 × 106 resolution setting and automatic gain control target of 106) will be followed by 10 data-dependent MS/MS spectra (automatic gain control target, 5,000; threshold, 3,000) in the linear ion trap from the 10 most abundant ions. Selected ions will be dynamically excluded for 30 s. Singly charged ions were excluded from MS/MS analysis. For β2AR ubiquitination site identification, MS/MS spectra were searched using the Sequest algorithm (36) with a variable mass addition of 114.0429 Da on lysines to match the diglycine signature (37). Oxidation of methionines (+15.9949 Da) was used as a variable modification and carbamidomethylation of cysteines (+57.0215) as a fixed modification. Spectral matches corresponding to β2AR ubiquitination sites were validated based on the mass accuracy (<5 ppm) of the precursor ion and manual inspection of the MS/MS spectra.

RESULTS AND DISCUSSION

Agonist-activated β2ARs Are Degraded by Lysosomal and Nonproteasomal Proteases—Both lysosomal and proteasomal inhibitors are known to increase β2AR levels and prevent degradation, as determined by radioligand binding with [125I]CYP (16, 38, 39). To further define these effects of lysosomal and proteasomal inhibitors, we determined the subcellular distribution of β2ARs by immunostaining with the β2AR-specific antibody H-20 after prolonged Iso stimulation. Under unstimulated conditions, FLAG-β2AR stably expressed in HEK-293 cells are localized at the plasma membrane, and there is no colocalization with the lysosomal marker protein, LAMP2 (Fig. 1A). As reported previously (17, 18), lysosomal localization of the β2AR is detected after 6 h of Iso stimulation in these cells (Fig. 1A), and despite the redistribution, the total receptor level is not significantly different from that in unstimulated cells. On the other hand, after 24 h of Iso stimulation, there is a dramatic reduction in β2AR levels compared with unstimulated cells by immunostaining with the anti-β2AR antibody (Fig. 1B). Additionally, inhibition of lysosomal proteases during prolonged Iso stimulation resulted in a marked stabilization of β2ARs in the lysosomal compartments (Fig. 1B). On the other hand, the increased levels of β2AR that resulted from prolonged treatment with both Iso and the proteasomal inhibitor MG132 showed little overlap with LAMP2-positive lysosomes but predominantly localized in nonlysosomal vesicles and perinuclear regions (Fig. 1B). Moreover, some receptor protein is detected
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**FIGURE 1.** Effects of proteasomal and lysosomal inhibitors in regulating cellular levels and distribution of agonist-activated β₂ARs. A, HEK-293 cells stably transfected with FLAG-β₂AR were stimulated with vehicle (nonstimulated, NS) or Iso for 6 h, and the cells were processed for confocal microscopy (Zeiss LSM510 META) as described under “Experimental Procedures.” β₂AR immunostained with anti-β₂AR H-20 antibody is shown in the green channel, and the lysosomal marker protein LAMP2 is shown in red. Merged images of the two channels are shown where yellow indicates colocalization. The data shown are from one of three independent experiments with identical results. The scale bars in all confocal panels represent 10 μm.

B, immunostaining of the β₂AR and LAMP2 was performed as in A after 24 h of Iso stimulation. The proteasomal inhibitor MG132 and lysosomal inhibitor leupeptin were added in the indicated samples along with Iso. The top row of confocal images display a group of cells, and the bottom panels display single-cell images for each condition as acquired with a 100× objective. The data shown are from one of three independent experiments with identical results.

C, HEK-293 cells stably transfected with FLAG-β₂AR were stimulated with vehicle or Iso for 24 h. The proteasomal inhibitor MG132 and lysosomal inhibitor leupeptin were added in the indicated samples. Equal protein amounts (μg) of lysate samples were used for immunoprecipitating (IP) the β₂AR with M2 anti-FLAG affinity gel, and the amount of receptor was assessed by Western blotting with anti-β₂AR H-20 antibody. The actin levels in the lysate samples are shown below.

D, the bands corresponding to mature receptor protein in C from four independent experiments were quantified, normalized to actin, and plotted as bar graphs. ***, p < 0.001 versus NS, one-way ANOVA, Bonferroni post test. NS, nonstimulated condition.

E, bar graphs were plotted as in D; however, the immature sharp β₂AR bands were quantified. ***, p < 0.001 versus NS and Iso; **, p < 0.01 versus Leu; *, p < 0.05 Leu + Iso, and MG132 only, one-way ANOVA, Bonferroni post test.

F, confocal images in both rows show the immunostaining of calreticulin (a marker for the endoplasmic reticulum) in the red channel and β₂AR in the green channel in cells treated with MG132 and Iso for 24 h. G and H, bar graphs represent the means ± S.E. of β₂AR degradation measured by [125I]CYP radioligand binding. ***, p < 0.001; **, p < 0.01 versus Iso-treated samples, one-way ANOVA, Bonferroni post test.
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at the plasma membrane, suggesting that even under the continuous presence of 10 $\mu$M Iso, receptor internalization is inhibited in the MG132-treated cells. MG132 is a generic inhibitor that blocks proteasomal degradation; this affects many cellular pathways including endocytosis, leading to a marked decrease in receptor internalization. Therefore, a major effect of 26 S proteasomal inhibition on $\beta_2$AR levels is from a general disruption of trafficking pathways.

We next analyzed whether both proteasomal and lysosomal inhibitors stabilize full-length mature glycosylated $\beta_2$AR proteins by immunoblotting. When we immunoprecipitated FLAG-$\beta_2$ARs from stably transfected HEK-293 cells with anti-FLAG affinity agarose and probed with anti-$\beta_2$AR H-20 antibody, we detected a 50% decrease in full-length $\beta_2$ARs upon 24 h of Iso stimulation (Fig. 1, C and D). The mobility of full-length mature receptor coincides with that of phosphorylated $\beta_2$ARs (18). When leupeptin was added along with Iso, a complete stabilization of full-length $\beta_2$ARs was observed (Fig. 1, C and D). By contrast, FLAG-IPs isolated from Iso-stimulated cells treated with MG132 showed an $\sim$50–60% decrease in the full-length receptor band similar to the decrease in cells treated with Iso alone (Fig. 1, C and D). However, a marked increase in immature receptor, mostly corresponding to newly synthesized $\beta_2$AR appearing as sharp bands, was observed (Fig. 1, C and E). As seen in the confocal images of MG132-treated cells (Fig. 1B), Iso stimulation engenders $\beta_2$AR trafficking to the perinuclear region, suggesting that MG132 arrests the $\beta_2$AR in the ER compartments. Previous studies have indicated that proteasomes mediate degradation of newly synthesized misfolded GPCRs at the ER (40, 41). Despite attempting with two different anti-proteasomal subunit antibodies, we were unable to obtain a specific signal by immunostaining for proteasomes in these cells. To test whether MG132 treatment traps the immature receptors in the ER as has been reported for other GPCRs, we immunostained these cells with an antibody that recognizes the ER marker protein calreticulin. As shown in the panels in Fig. 1F, in MG132-treated cells, a significant amount of $\beta_2$AR is colocalized with calreticulin. However, these two proteins do not colocalize in unstimulated, Iso-treated, or leupeptin-treated cells (supplemental Fig. S1). These data suggest that upon 26 S proteasomal inhibition with MG132, immature $\beta_2$ARs that have escaped endoplasmic reticulum associated protein degradation are localized in the ER compartments. In contrast to these results obtained with MG132, the $\beta_2$ARs of cells treated with Iso in the presence of leupeptin reside in late endosomes/lysosomes (Fig. 1B). Taken together with the observation that leupeptin treatment rescues full-length $\beta_2$ARs from Iso-induced degradation (Fig. 1C), these immunofluorescence data suggest that lysosomes are the primary site of degradation of $\beta_2$ARs upon Iso stimulation.

To further verify that MG132 treatment stabilizes mostly newly synthesized immature receptors, we determined the total receptor levels by $^{125}$I[CYP radioligand binding in Iso-treated cells with and without the protein synthesis inhibitor cycloheximide (CHX, 50 $\mu$g/ml) treatment, combined with either leupeptin or MG132. Leupeptin treatment inhibited degradation of the $\beta_2$AR to the same extent with or without CHX (Fig. 1G and supplemental Table S1). On the other hand, MG132 inhibited receptor degradation only in the absence of CHX (Fig. 1H and supplemental Table S1). In the presence of CHX, Iso-stimulated degradation of the $\beta_2$AR was identical with or without MG132 treatment (Fig. 1H and supplemental Table S1). This suggests that MG132 stabilizes mostly newly synthesized $\beta_2$ARs. Because $^{125}$I[CYP can bind even to the truncated and immature GPCRs that are “rescued” by MG132 through its ability to block the ER-associated degradation, a significant increase in receptor levels is detected in the absence of CHX (16, 42). Taken together, these data strongly suggest that the lysosomal pathway predominates in degrading agonist-activated $\beta_2$ARs, and 26 S proteasomes are not the primary means of degrading agonist-activated $\beta_2$ARs. Rather the proteasomal pathway plays a major role in the quality control of newly synthesized $\beta_2$ARs as described for other GPCRs (40–42).

Lysosomal Trafficking Is Correlated with $\beta_2$AR Ubiquitination—Lysosomal targeting and subsequent degradation of the activated $\beta_2$AR is dependent on agonist-induced ubiquitination of the receptor (16–18, 26). The human $\beta_2$AR contains 16 total and 14 intracellular lysine residues in its primary sequence (Fig. 2A). Mutation of all of the lysines to arginines (mutant receptor, 0K-$\beta_2$AR (43) prevents the covalent attachment of ubiquitin as well as receptor degradation (16). As shown in Fig. 2B, 0K-$\beta_2$AR-mYFP, internalizes into endosomes at 20 min and 1 h of Iso stimulation, but at 6 h, only a negligible portion of the internalized 0K-$\beta_2$AR colocalizes with the LysoTracker$^\text{TM}$ dye (Molecular Probes) that labels late endosomes/lysosomes, and most of the 0K-$\beta_2$AR is recycled to the plasma membrane (Fig. 2B). In contrast, a substantial proportion of internalized WT $\beta_2$AR-mYFP colocalizes with the LysoTracker$^\text{TM}$ and is sorted into late endosomes/lysosomes after 6 h of Iso stimulation (Fig. 2B), although both WT and 0K $\beta_2$ARs internalize into endosomes at 20-min and 1-h time points to a similar extent (Fig. 2B).

Lysosomal Trafficking of Iso-stimulated $\beta_2$AR Involves Ubiquitination in Two Intracellular Domains—The altered trafficking of 0K-$\beta_2$AR could be attributed to the lack of ubiquitin moieties covalently attached to the receptor, because WT $\beta_2$ARs that are deubiquitinated show negligible lysosomal degradation and accelerated recycling (18). To gain mechanistic insights about the linkage between receptor ubiquitination and lysosomal trafficking, we sought to identify the domains within the $\beta_2$AR that are targeted for ubiquitination. To define the ubiquitination sites in the $\beta_2$AR, we reintroduced lysines into the global Lys-to-Arg mutant 0K-$\beta_2$AR-either in individual cytoplasmic loops (L) or in the carboxyl-terminal tail (CT) and thereby generated four amino-terminal FLAG epitope-tagged $\beta_2$AR mutants: L1 $\beta_2$AR (containing lysine 60 only), L2 $\beta_2$AR (containing lysines 140, 147, and 149), L3 $\beta_2$AR (containing lysines 227, 232, 235, 263, 267, 270, and 273), and CT $\beta_2$AR (containing lysines 348, 372, and 375).

To assess signaling efficacy of the $\beta_2$AR mutants, we expressed them individually in HEK-293 cells stably expressing the FRET reporter ICUE2 to facilitate quantitation of the cAMP response to Iso stimulation. As shown in Fig. 3A, each $\beta_2$AR mutant was able to induce robust cAMP signals, much above the levels induced by endogenously expressed receptors (represented by pCDNA3 transfection) in these live cell reporter
assays. The cell surface expression levels of all of the mutants were comparable with that of the WT β2AR as determined by (−)[3H]CGP-12177 binding and were ~20–30-fold in excess of endogenous levels of β2AR in these cells. Cell surface receptor expression levels (pmol/mg) as determined by (−)[3H]CGP-12177 were: WT, 2.9 ± 0.1; 0K, 1.8 ± 0.1; L1, 3.3 ± 0.5; L2, 5.8 ± 0.6; L3, 2.2 ± 0.4; CT, 2.8 ± 0.06; and pCDNA3, 0.1 ± 0.008. All of the lysine mutants were able to normally couple to Gs and induce cAMP production much like the WT β2AR (Fig. 3A). In these assays, the Iso dose-response curves of the mutants were not significantly different from that of the WT β2AR, thus suggesting that their functional capability toward second messenger signaling was unperturbed by these mutations.

The 0K-β2AR as well as its Lys add-back mutants were able to recruit GFP-β-arrestin2 within a minute of Iso stimulation in a manner similar to the WT β2AR (Fig. 3, B and C). To test the ability of each receptor mutant to undergo Iso-stimulated internalization, we measured the decrease in cell surface β2ARs after a 30-min Iso treatment (Fig. 3D and supplemental Fig. S2). All of the mutants internalized to an extent similar to or slightly higher than the WT receptor. Thus, β2AR lysyl to arginyl mutations do not appear to affect β-arrestin recruitment or β2AR internalization.

Although the different lysine add back mutants were indistinguishable in terms of β2AR internalization, they were easily distinguishable in terms of ubiquitination. Subsequent to cell stimulation by Iso, only the WT, L3, and CT β2ARs demonstrated ubiquitination, suggesting that only the lysines in the third loop and CT of the β2AR are targeted for ubiquitination (Fig. 4A). Although ubiquitination of the WT β2AR exceeded that of either the L3 or CT β2ARs, both the third cytoplasmic loop and the cytoplasmic tail of the β2AR undergo significant ubiquitination. Additionally, ubiquitination within even just one of these domains is sufficient to target the agonist-activated β2AR to the lysosomes; like the WT β2AR (Fig. 4B), both the L3 β2AR and CT β2AR displayed colocalization with LAMP2 after cells were stimulated with Iso for 6 h (Fig. 4B). In contrast, the 0K, L1, and L2 β2ARs were neither ubiquitinated (Fig. 4A) nor targeted to the lysosomes after prolonged cell stimulation with
That β2AR ubiquitination involves both the β2AR third intracellular loop and cytoplasmic tail is further demonstrated by studies of mutant β2AR degradation. As measured by radioligand binding, β2AR degradation in response to Iso was 35, 14, and 22, respectively, for the WT, L3, and CT β2ARs (Fig. 4C and supplemental Table S2). Thus, ubiquitination of both the third intracellular loop and the carboxyl tail of the β2AR appear to be responsible for efficient degradation of the receptor protein in the lysosomes.

**Direct Identification of Ubiquitinated Lysines within the β2AR by Mass Spectrometry**—To further our understanding of the mechanisms of β2AR ubiquitination and lysosomal trafficking, we sought to identify the actual sites of ubiquitin conjugation by subjecting the ubiquitinated β2AR to online LC-MS/MS analysis. This approach has been successfully utilized for mapping ubiquitination sites on a variety of proteins (44–48) but thus far not reported for a GPCR. A HEK-293 cell line stably overexpressing WT β2AR (2.5 pmol/mg cellular protein) was used for preparing samples for mass spectrometry analysis. The stable cells were starved in serum-free medium overnight and treated with 10 mM Iso for 1 h prior to harvest. WT β2AR was isolated using alprenolol-Sepharose affinity resin and subjected to in-solution trypsin digestion, which cleaves the receptor proteins at the carboxyl termini of lysyl and arginyl residues. Trypsin also cleaves ubiquitin between Arg-74 and Gly-75 and leaves diglycine motifs (Gly-75–Gly-76) covalently attached to the modified Lys residues (37). This results in signature peptides with a Lys-Gly-Gly branch and a mass shift of 114.0429 Da that allows the identification of Lys residues as ubiquitin conjugation sites. Using this approach, five lysyl residues were identified.
fied on the β2AR as sites of ubiquitination: Lys-263, Lys-270, Lys-348, Lys-372, and Lys-375 (Fig. 5A). Of these lysyl residues, the first two are located in the third intracellular loop, and the other three are located in the carboxyl tail of the β2AR. The representative MS/MS spectra for β2AR ubiquitination are shown in Fig. 5 (B and C) and supplemental Fig. S3. Shorter peptide sequences with ubiquitin signatures were retrieved from the third loop than the carboxyl tail region. This could be attributed to the abundance of tryptic cleavage sites in the third loop (seven lysyl and six arginyl residues within a stretch of 54 total residues). We did not detect ubiquitinated receptor peptides of the β2AR in samples isolated from unstimulated cells. Therefore, the sites identified correspond to ubiquitination of the β2AR promoted by Iso stimulation. These sites are also concordant with the findings obtained by the molecular approaches described above that the third loop and carboxyl tail of the β2AR are targeted for ubiquitination upon Iso stimulation.

Iso stimulation of the β2AR can also induce receptor phosphorylation by both protein kinase A and G protein-coupled
FIGURE 5. Direct identification of ubiquitinated lysines in the β2AR by LC-MS/MS. β2ARs were purified from HEK-293 cells stably expressing β2ARs using an alprenolol-Sepharose affinity matrix as described under "Experimental Procedures," proteolyzed with trypsin, and analyzed by mass spectrometry using a Thermo Scientific LTQ Orbitrap XL mass spectrometer. A, list of peptide sequences containing the ubiquitination sites identified in this study. The ubiquitinated sites are highlighted in red, and the amino acid positions within the β2AR sequence are listed. B and C show two representative annotated MS/MS fragmentation spectra for ubiquitinated peptides SSLK<sup>Ub</sup>AYNGYSSNGNTGEQSGYHVEQK and AYGNGYSSNGNTGEQSGYHVEQK<sup>Ub</sup>ENK, respectively. The peptide sequences are shown at the top of the MS/MS spectra with ubiquitinated residues highlighted in red. The peak heights are the relative abundances of the corresponding fragmentation ions, with the annotation of the identified matched amino terminus-containing ions (b ions) in blue and the carboxyl terminus-containing ions (y ions) in red. For clarity, only the major identified peaks are labeled. The spectra are representative MS/MS fragmentation spectra from one of three independent experiments.
Mapping β₂AR Ubiquitination

receptor kinases (GRks) on the third intracellular loops and carboxyl tail (49). Most ubiquitinated peptides identified in this study contain putative phosphorylation sites. β₂AR phosphorylation precedes and is required for efficient ubiquitination, because a β₂AR mutant deficient in phosphorylation is not ubiquitinated (16). Similar correlation between receptor phosphorylation and ubiquitination is also reported for the yeast GPCRs STE2 and STE3 and could be prevalent in other membrane receptors (50–53). β₂AR is also hydroxylated at Pro-382 and Pro-395 on the carboxyl tail of the β₂AR, which is detectable without agonist activation (54). The two proline hydroxylation sites are in a tryptic peptide (residue 373–404) where we identified β₂AR Lys-375 ubiquitination. To examine whether these different post-translational modifications occur within the same peptides, we performed database searches using parameters, which allow detection of all three modifications: ubiquitinated Lys (+114.0429 Da), hydroxylated Pro (+15.9949 Da), and phosphorylated Ser, Thr, or Tyr (+79.9663 Da). We did detect peptides containing signals for both receptor phosphorylation and proline hydroxylation (data not shown). To our surprise, we did not detect a ubiquitination site that also has a phosphorylation or proline hydroxylation modification. One explanation for the failure to detect the coexistence of different modifications on a single peptide is low stoichiometry. Alternatively, receptors that are tagged for degradation with ubiquitin could be rapidly dephosphorylated and dehydroxylated, whereas prolyl hydroxylation that occurs basally could be preserved after receptor phosphorylation until receptors are ubiquitinated. Such differential kinetics of the various post-translational modifications occurring on the β₂AR could prevent their simultaneous detection.

Although recent crystal structures of the β₂AR have revealed additional structural information on the transmembrane helices, atomic details on the entire third intracellular loop are limited because of the modifications introduced to facilitate receptor crystallization (55–58). Nonetheless, Lys-263, identified as a ubiquitination site in the third loop, is close to Glu-268, which forms a salt bridge with Arg-131 located in transmembrane helix 3 and constitutes the ionic lock described in the crystal structures; disruption of this ionic lock occurs during receptor activation (59, 60). Thus, agonist-induced ubiquitination at this site might correlate with conformational changes and/or receptor activation and coincide with disruption of the ionic lock. On the other hand, structural insights from β₂AR crystals cannot account for the role of the carboxyl-terminal tail, because the crystallization studies employed a mutant β₂AR that was truncated at the carboxyl-terminal tail and lacked residues 366–411 of the full-length β₂AR (56, 61). However, previous studies with mutant β₂ARs have demonstrated that both the third intracellular loop and the carboxyl-terminal tail of the β₂AR are vital for binding to G proteins and β-arrestins (62–64). Additionally, studies conducted with chimeric receptors in cardiac myocytes predict that both the third intracellular loop domain and the carboxyl tail regions are involved in spontaneous activation of the β₂AR (65). Our studies further reinforce the idea that timely ubiquitination of the β₂AR in these two domains could be critical for signal desensitization by tagging activated receptors for lysosomal degradation.

In conclusion, our studies demonstrate that agonist-activated β₂-ARs are degraded in the lysosomes and that 26 S proteasomes do not play a central role in this process. Additionally, receptor ubiquitination is required for the lysosomal degradation of agonist-activated receptors. Our studies based on both molecular and proteomics approaches indicate that ubiquitination of the β₂AR occurs on both the third intracellular loop and carboxyl tail of the β₂AR. These two domains are critical in defining the magnitude, extent, and cellular destinations of downstream signaling of the β₂AR via both G protein and β-arrestin-dependent pathways. Future studies should reveal whether ubiquitination of the β₂AR, while dictating intracellular trafficking to the lysosomes, also regulates its dynamic protein-protein interactions in signalosomes, thus orchestrating the translocation and/or compartmentalization of β₂AR signaling complexes.

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