THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 23, Issue of June 10, pp. 22124–22134, 2005

Printed in U.S.A.

The Glucagon-like Peptide-2 Receptor C Terminus Modulates β-Arrestin-2 Association but Is Dispensable for Ligand-induced Desensitization, Endocytosis, and G-protein-dependent Effector Activation*[S]

Received for publication, January 4, 2005, and in revised form, March 14, 2005
Published, JBC Papers in Press, April 6, 2005, DOI 10.1074/jbc.M500078200

Jennifer L. Estall‡§¶, Jacqueline A. Koehler§‖, Bernardo Yusta§, and Daniel J. Drucker‡§‡‡**

*From the Departments of ‡Laboratory Medicine and Pathobiology, and **Medicine, University of Toronto, §The Banting and Best Diabetes Centre, Toronto General Hospital, University of Toronto, Toronto M5G 2C4, Canada*

Classic models of receptor desensitization and internalization have been largely based on the behavior of Family A G-protein-coupled receptors (GPCRs). The glucagon-like peptide-2 receptor (GLP-2R) is a member of the Family B glucagon-secretin GPCR family, which exhibit significant sequence and structural differences from the Family A receptors in their intracellular and extracellular domains. To identify structural motifs that regulate GLP-2R signaling and cell surface receptor expression, we analyzed the functional properties of a series of mutant GLP-2Rs. The majority of the C-terminal receptor tail was dispensable for GLP-2-induced cAMP accumulation, ERK1/2 activation, and endocytosis in transfected cells. However, progressive truncation of the C terminus reduced cell surface receptor expression, altered agonist-induced GLP-2R trafficking, and abrogated protein kinase A-mediated heterologous receptor desensitization. Elimination of the distal 21 amino acids of the receptor was sufficient to promote constitutive receptor internalization and prevent agonist-induced recruitment of β-arrestin-2. Site-directed mutagenesis identified specific amino acid residues within the distal GLP-2R C terminus that mediate the stable association with β-arrestin-2. Surprisingly, although the truncated mutant receptors failed to interact with β-arrestin-2, they underwent homologous desensitization and subsequent resensitization with kinetics similar to that observed with the wild-type GLP-2R. Our data suggest that, although the GLP-2R C terminus is not required for coupling to cellular machinery regulating signaling or desensitization, it may serve as a sorting signal for intracellular trafficking. Taken together with the previously demonstrated clathrin and dynamin-independent, lipid-raft-dependent pathways for internalization, our data suggest that GLP-2 receptor signaling has evolved unique structural and functional mechanisms for control of receptor trafficking, desensitization, and resensitization.

Glucagon-like peptide-2 (GLP-2)[1] is a peptide hormone released from intestinal L-cells in response to nutrient ingestion (1). GLP-2 action is initiated following binding to the glucagon-like peptide-2 receptor (GLP-2R), a Family B G-protein-coupled receptor (GPCR) (2) that has been localized to human enteroendocrine cells (3), murine enteric neurons (4), and both human and rodent myofibroblasts (5) within the gastrointestinal tract. GLP-2R signaling directly inhibits apoptosis in transfected cells (6–8) and maintains gastrointestinal mucosal integrity due to its ability to stimulate proliferation and inhibit cell death in the intestinal crypt compartment (9–11). The cytoprotective effects of GLP-2, demonstrated in rodent models of intestinal injury (for review, see Ref. 11), have generated interest in the use of long-acting GLP-2 analogs for the treatment of human intestinal disease.

Because long-acting GPCR agonists promote persistent receptor activation, concerns have been raised regarding possible down-regulation of receptor signaling. The mechanisms regulating GPCR desensitization, down-regulation, and intracellular trafficking have been extensively characterized for members of the Family A rhodopsin-like GPCRs (for review, see Ref. 12), but few studies have addressed whether these cellular processes are conserved for the structurally distinct Family B GPCRs. Furthermore, existing reports suggest that this family of GPCRs, which includes the highly homologous glucagon, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide receptors (13), may have evolved distinct mechanisms to regulate receptor signaling at the cellular level (14–16). In this study, we attempted to delineate the mechanisms regulating GLP-2R desensitization and identify structural motifs within C-terminal GLP-2R domains that link the

* The work was supported in part by operating grants from the Canadian Institutes of Health Research (CIHR), the National Cancer Institute of Canada, and the Ontario Research and Development Challenge Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains additional text.

¶ Supported by a CIHR Canadian Graduate Scholarship Doctoral Award and a grant from the Banting and Best Diabetes Centre/Novo-Nordisk.

‖ Supported by a postdoctoral fellowship award from the Banting and Best Diabetes Centre and the McLaughlin Center for Molecular Medicine.

‡‡ Supported by a Canada Research Chair in Regulatory Peptides. To whom correspondence should be addressed: Toronto General Hospital Banting and Best Diabetes Centre, 200 Elizabeth St., MBRC4R-402, Toronto M5G 2C4, Canada. Tel.: 416-340-4125; Fax: 416-978-4108; E-mail: d.drucker@utoronto.ca.

[1] The abbreviations used are: GLP-2, glucagon-like peptide-2; GLP-2R, glucagon-like peptide-2 receptor; GPCR, G-protein-coupled receptor; FBS, fetal bovine serum; FK, forskolin; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; BHK, baby hamster kidney fibroblast; DMEM, Dulbecco's modified Eagle's medium; WT, wild type; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; GFP, green fluorescent protein; PKA, protein kinase A; PKC, protein kinase C; MAPK, mitogen-activate protein kinase; JNK, c-Jun N-terminal kinase.

receptor to downstream signaling pathways and direct interaction of the receptor with intracellular sorting machinery.

It is generally accepted that intracellular GPCR domains, most prominently the C terminus and 3rd intracellular loop, regulate receptor desensitization, endocytosis, and intracellular fate by facilitating association of the receptor with various cellular proteins. Receptor activation promotes phosphorylation of these intracellular domains, by either second messenger kinases or G-protein receptor kinases. G-protein receptor kinase-mediated phosphorylation facilitates β-arrestin binding, promoting uncoupling of the receptor from G-proteins and endocytosis via clathrin-coated pits (for more detailed review, see Ref. 12). We have previously shown that the GLP-2R undergoes rapid and persistent ligand-induced (homologous) desensitization accompanied by clathrin- and dynamin-independent, lipid-raft-dependent receptor endocytosis (17). Internalized GLP-2Rs are then rapidly trafficked out of lipid-raft-derived endosomes, where they converge on the early endosomal and perinuclear recycling compartments. Identification of this alternative trafficking pathway led us to hypothesize that unique structural domains within the GLP-2R may be responsible for directing the receptor through this intracellular fate. Moreover, these structural domains may be conserved for other members of the GPCR superfamily.

To address our hypothesis, we used mutagenesis to identify regions within the GLP-2R C terminus that regulate receptor coupling to downstream signaling pathways or ligand-induced receptor trafficking. Our study demonstrates that the GLP-2R C terminus, which modulates the stable association with β-arrestin-2, is dispensable for G-protein-coupled signaling, homologous desensitization, and receptor endocytosis. However, the GLP-2R C terminus is required for retention of un-liganded receptor on the plasma membrane and may direct intracellular trafficking of internalized receptors. Taken together, our data suggest that this intracellular domain and its interacting proteins likely govern alternative, currently unidentified, functions in the regulation of GLP-2R activity.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium was purchased from HyClone (Logan, UT); fetal bovine serum (FBS), G418, and antibiotics were from Invitrogen. 3-Isobutyl-1-methylxanthine, anti-FLAG (M1 or M2 as indicated) antibody, anti-mouse IgG-agarose, mouse IgG, 2,2′-azinobis(3-ethylbenthiazoline-6-sulfonic acid reagent, phorbol 12-myristate 13-acetate, forskolin (FK), protease inhibitor mixture, and phosphatase inhibitor mixture I were all obtained from Sigma. Dithiobis(succinimidylpropionate) was purchased from Pierce Biotechnology (Rockford, IL). The anti-β-arrestin antibody, Ro-31-8425, *N*-[2-((*p*-bromocinnamyl)-amino)ethyl]-5-isoquinolinesulfonamide (H-89), and pertussis toxin were purchased from Calbiochem. Phospho-p44/42 ERK1/2 antibody was obtained from Cell Signaling Technology (Pickering, Ontario, Canada). The antibody Hsp90 was purchased from BD Transduction Laboratories (BD Biosciences, Mississauga, Ontario, Canada). The donkey antibody anti-mouse-Cy3 was obtained from Jackson Laboratories (West Grove, PA). Anti-mouse- and anti-rabbit-horseradish peroxidases (HRP) were purchased from Amersham Biosciences. Human glucagon-like peptide-2 (GLP-2) was from Bachem (Torrance, CA).

Plasmids—The expression vector pcDNA3.1 was obtained from Invitrogen. The pcDNA3.1-human GLP-2R (GLP-2R) plasmid was a gift from NPS Pharmaceuticals (Salt Lake City, UT). The wild-type (WT) FLAG epitope-tagged human GLP-2R (FLAG-GLP-2R) cDNA was generated as previously described (17). The expression vectors encoding GFP-tagged rat β-arrestin-2 (β-arrestin-2-GFP) and untagged wild-type rat β-arrestin-2 were kind gifts from Dr. M. G. Caron (Duke University Medical Center, Durham, NC) and Dr. S. Ferguson (Robarts Research Institute, London, Ontario, Canada), respectively.

Generation of Mutant GLP-2R Constructs—The truncated mutant human GLP2R receptor cDNAs were generated by PCR with primers that introduced a stop codon and an XhoI site following the codons encoding amino acids 553 (WT), 503 (Δ504), 469 (Δ470), 449 (Δ450), or 443 (Δ444), utilizing pcDNA3.1-hGLP2R as a template. To generate the Δ4445A mutant, five alanine residues were introduced 3′ to amino acid 443, followed by a stop codon. Single and multiple point mutant GLP-2Rs were also generated by PCR. The PCR products were cloned into the FLAG-tagged-hGLP2R (pcDNA3.1) plasmid previously described (17) (see Supplementary Material for details and primer sequences). The authenticity of the WT and mutant FLAG-GLP-2R cDNAs was verified by automated sequencing (Core Molecular Biology Facility, York University, Ontario, Canada).

Cell Culture—Baby hamster kidney fibroblasts (BHK-21), human colon cancer epithelial cells (DLD-1), and human cervical cancer cells (HeLa) were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units of penicillin/100 μg of streptomycin and either 10% (BHK and HeLa) or 5% (DLD-1) FBS. DLD-1 cells stably expressing either the WT or mutant GLP-2Rs were generated following transfection of linearized WT or mutant GLP-2R constructs using FuGENE™ (Roche Diagnostics) and selection with 1.2 mg/ml G418. Pools of DLD-1 clones, stably expressing the aforementioned plasmids, were used for further experimental analysis and maintained in media supplemented with 0.5 mg/ml G418. Transient transfections of BHK, DLD-1, or HeLa cells were carried out using FuGENE™ according to the manufacturer's protocol.

Measurement of Cell Surface and Total Cellular GLP-2 Receptor Levels—To assess levels of cell surface receptor, cells transiently transfected with either WT or mutant FLAG-GLP-2R cDNA constructs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at 4 °C. Fixed cells were incubated at room temperature with FLAG (M1) antibody (1:2000) diluted in DMEM containing 10% FBS and 1 mM $CaCl_2$ for 1 h, washed, and then incubated with anti-mouse-HRP (1:1000) in DMEM (10% FBS/1 mM $CaCl_2$) for 1 h. Cell-associated horseradish peroxidase activity was quantified by incubation at room temperature with 2,2′-azinobis(3-ethylbenthiazoline-6-sulfonic acid) (1 mg/ml) in 0.1 M sodium citrate, 0.1 M Na_2HPO_4, pH 4.0, and 1 μl of 30% H_2O_2/ml. The reaction was stopped with 0.5% SDS/0.1% sodium azide, and the absorbance of the HRP reaction product was measured at 405 nm. HRP background absorbance from cells transfected with either untagged receptor cDNA or pcDNA3.1 was subtracted to determine final absorbance values.

To assess receptor internalization, transfected cells were incubated with agonist for the indicated periods of time prior to fixation. For assessment of cell surface receptor recovery following internalization, cells were incubated with agonist for 20 min at 37 °C, washed briefly with warm PBS, and then re-incubated in DMEM supplemented with serum for 30–120 min at 37 °C prior to fixation. To measure levels of total cellular FLAG-GLP-2R, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature following fixation and prior to incubation with the anti-FLAG (M1) antibody.

Measurement of Total cAMP Accumulation—24 h following transfection, cells were stimulated with 0–100 nM GLP-2 diluted in media containing 100 μM 3-isobutyl-1-methylxanthine for 10 min at 37 °C. Ice-cold ethanol was added to each well (final concentration of 77%) to stop the reaction. Following an overnight incubation at −20 °C, the cAMP content of the ethanol extracts was measured by radioimmunoassay (Biomedical Technologies, Stoughton, MA).

Immunofluorescence Microscopy—BHK cells, grown in chamber slides (Nalge Nunc, Int., Naperville, IL) and transiently transfected with WT or mutant FLAG-GLP-2Rs, were incubated at 4 °C with anti-FLAG (M1) antibody (1:350) for 1 h in DMEM buffered with 25 mM HEPES (Invitrogen) and supplemented with 10% FBS and 1 mM $CaCl_2$. After washing with PBS plus 1 mM $CaCl_2$, cells were incubated at 37 °C for 60 min in the absence (control) or presence of 10 nM GLP-2 diluted in DMEM containing 10% FBS and 1 mM $CaCl_2$, to induce receptor endocytosis, and immediately fixed with 4% paraformaldehyde plus 1 mM $CaCl_2$ at 4 °C for 30 min. Following permeabilization with 0.2% Triton X-100/1 mM $CaCl_2$ and blocking with 10% horse serum, 1% bovine serum albumin, 1 mM $CaCl_2$ and 0.02% azide in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.6/136 mM NaCl) for 1 h at room temperature, the cells were incubated at room temperature with donkey anti-mouse-Cy3 (1:1000) antibody diluted in 5% bovine serum albumin/1 mM $CaCl_2$ in TBS. The slides were mounted using anti-fade mounting media (DAKO Diagnostics Canada Inc., Ontario, Canada), and visualized using a Zeiss LSM 510 confocal microscope with a 63× oil immersion objective (Carl Zeiss, Thornwood, NY). For co-localization of FLAG-GLP-2R and β-arrestin-2-GFP, cells were transiently co-transfected with β-arrestin-2-GFP and either WT or mutant FLAG-GLP-2Rs prior to treatment and staining. Due to the lower cell surface receptor levels of the truncated GLP-2R mutants, detector gain was increased to visualize and document immunofluorescence associated with the labeled mutant receptors.

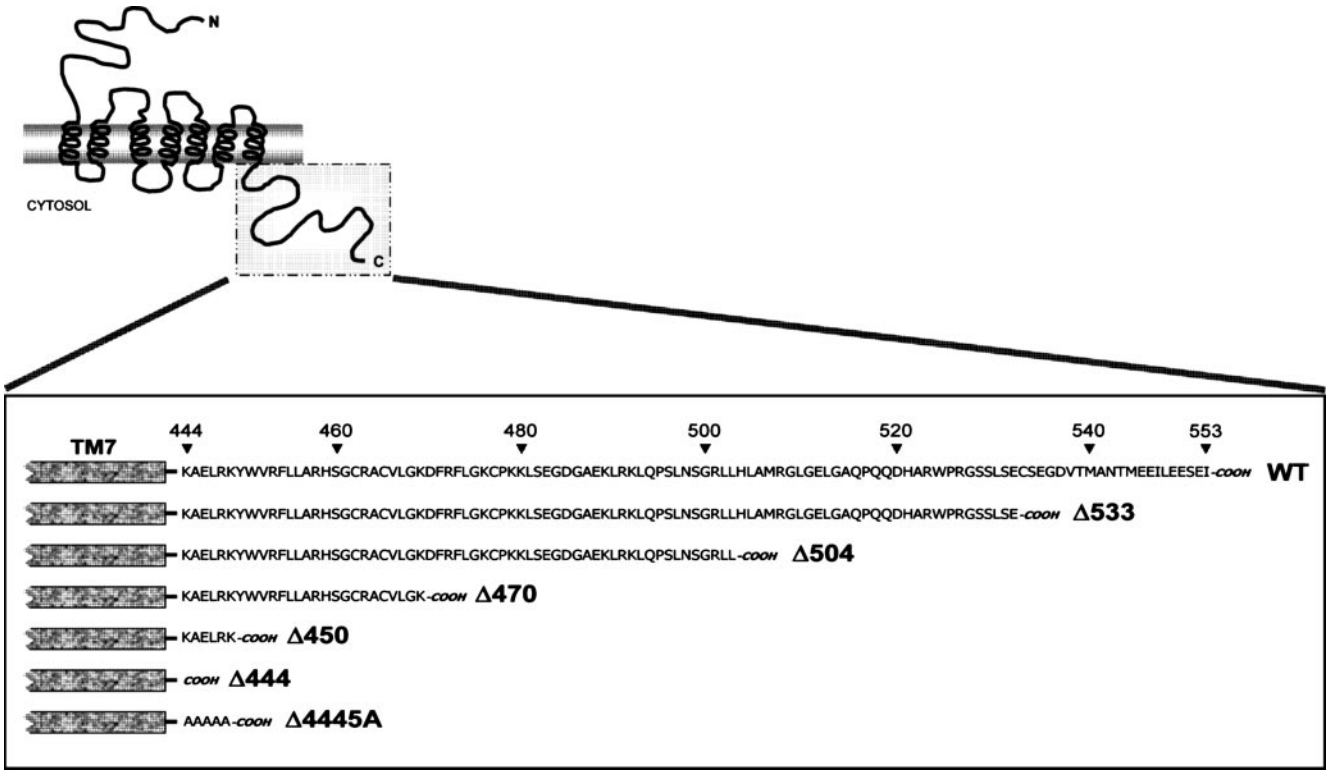

FIG. 1. **Schematic representation of the truncated mutant GLP-2R constructs.** The amino acid sequence (residue numbers shown above sequence) of the entire GLP-2R C terminus (*WT*) is shown downstream of the predicted 7th transmembrane domain (*TM7*). The different truncated mutant receptors were constructed using PCR as described under "Experimental Procedures" to introduce stop codons at specific positions, thus shortening the C terminus as indicated. The Δ4445A mutant receptor was constructed to mimic the Δ450 mutant receptor in length but does not contain any GLP-2R-specific C-terminal amino acid sequence. The Δ444 mutant is missing the entire predicted C-terminal tail.

Immunoprecipitation and Western Blot Analysis of FLAG-GLP-2R—BHK cells transfected with untagged wild-type β-arrestin-2 and either WT or mutant FLAG-GLP-2R constructs were washed twice with PBS and treated for 0–60 min with 10 nM GLP-2 in serum-free DMEM buffered with 25 mM HEPES. Cellular proteins were then cross-linked for 30 min at room temperature while rocking following the addition of freshly prepared dithiobis(succinimidylpropionate) dissolved in Me_2SO (1.25 mM final concentration). Plates were placed on ice and washed twice for 5 min with cold media supplemented with 10% FBS. Following two washes with ice-cold PBS, cells were lysed on ice for 30 min in ice-cold lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, protease inhibitor mixture, phosphatase inhibitor mixture, 5 mM NaF, and 500 μM sodium orthovanadate (radioimmune precipitation assay buffer). The protein concentration of cleared lysates was determined using the BCA Protein assay kit (Pierce). 500 μg of each lysate was pre-cleared for 1 h with anti-mouse agarose beads linked to 1 μg of nonspecific mouse IgG and then incubated overnight at 4 °C with anti-mouse agarose beads linked to 2 μg of anti-FLAG (M2) antibody. Immunoprecipitates were washed four times with radioimmune precipitation assay buffer and treated with 10× reducing sample buffer for 1 h at 37 °C prior to resolving by SDS-PAGE and transfer to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). In parallel, 40 μg of each whole cell lysate was resolved to assess β-arrestin-2 expression levels. Blots were blocked in 5% milk in PBS containing 0.2% Tween 20 and incubated at room temperature with anti-β-arrestin (1:1000). Antigen-antibody complexes were visualized with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence (ECL) kit (Amersham Biosciences). Blots were stripped and reprobed with anti-FLAG (M1) (1:1000) to monitor the levels of precipitated WT and mutant receptors.

Desensitization Protocol and Determination of Intracellular cAMP Content—To induce desensitization, stably transfected cells were incubated with DMEM containing 10 nM GLP-2, 20 μM forskolin, or vehicle (PBS), as indicated, for 20 min. Following a brief wash with warm PBS, the cells were allowed to recover in DMEM containing 5% FBS for the indicated periods of time. The cells were then re-stimulated with media containing 100 nM GLP-2 and 100 μM 3-isobutyl-1-methylxanthine for 10 min at 37 °C. The reaction was stopped by a quick wash with PBS at 4 °C followed by the addition of cAMP extraction buffer (77% EtOH in DMEM containing 100 μM 3-isobutyl-1-methylxanthine). The cAMP concentration in the ethanol extracts was assessed by radioimmunoassay as described above. To assess the effect of PKA inhibition on GLP-2R desensitization, cells were incubated in media containing 10 μM H-89 for 30 min prior to and throughout pretreatment and recovery periods.

Assessment of ERK1/2 Activation—HeLa cells transiently transfected with WT or mutant GLP-2R receptors were serum-starved overnight prior to stimulation with vehicle or 20 nM GLP-2 for 0–60 min, as indicated. Cells were lysed on ice in 50 mM Tris, pH 8.0, 150 mM NaCl, containing 1% Triton X-100, protease and phosphatase inhibitor cocktails, and 500 μM sodium orthovanadate. 20 μg of each cleared lysate was resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Following blocking for 1 h at room temperature in 5% bovine serum albumin in TBS containing 0.02% Tween 20, blots were incubated overnight at 4 °C with anti-phospho-p42/p44 ERK1/2 antibody (1:1000) diluted in blocking buffer. A primary antibody against Hsp90 (1:2000) was used as a loading control. Antigen-antibody complexes were visualized with a secondary antibody conjugated to horseradish peroxidase and ECL. To assess the roles of $G\alpha_{i/o}$ subunit activation and PKC in GLP-2R-dependent ERK1/2 activation, cells were preincubated with either 200 ng/ml pertussis toxin for 24 h or 1 μM Ro-31-8425 (Ro) for 1 h prior to and during stimulation with GLP-2.

Statistical Analysis—Statistical significance was assessed by one-way analysis of variance followed by the Bonferroni multiple comparison post-hoc test using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

RESULTS

The GLP-2R C Terminus Modulates Cell Surface Receptor Expression, But Not Adenylyl Cyclase Activation—Multiple protein domains and structural motifs within the C-terminal domains of GPCRs have been implicated in the control of receptor expression and signaling. To identify domains within the GLP-2R that regulate receptor signaling, trafficking, and desensitization, we constructed a series of mutant FLAG-

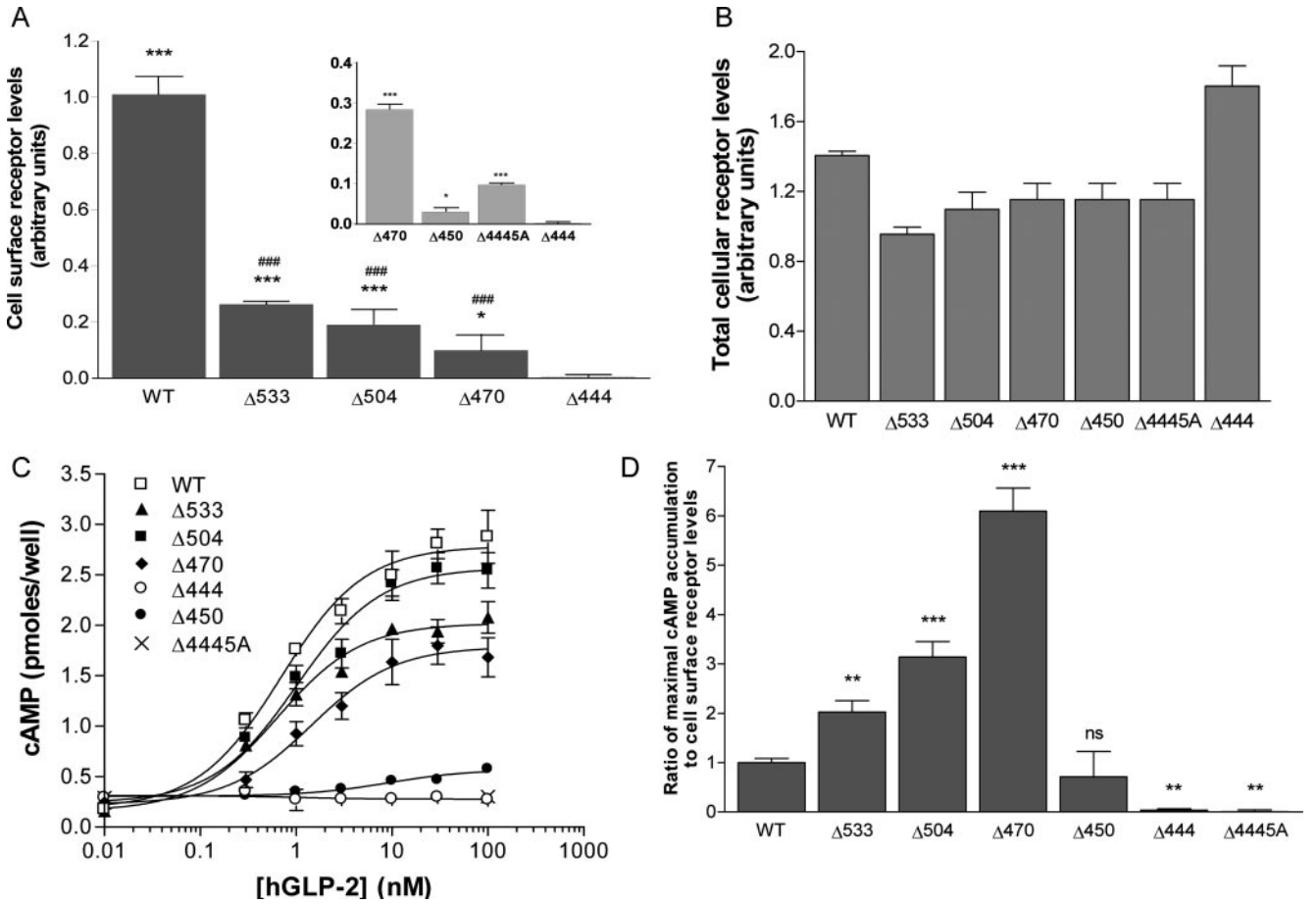

FIG. 2. **Progressive truncation of the hGLP2R C terminus results in decreased cell surface receptor expression, but enhanced GLP-2-induced cAMP production.** *A*, BHK cells transiently transfected with the same amount of FLAG-tagged wild-type (*WT*) or truncated mutant GLP-2Rs cDNAs were assayed for cell surface receptor expression (*A*) or total cellular receptor levels (*B*) using an HRP-linked immunosorbent assay, as described under "Experimental Procedures." The *inset* in *A* illustrates the low, but detectable, cell surface expression of the Δ450 and Δ4445A receptor mutants. Data are mean ± S.D. ($n = 3$) and are representative of four independent experiments. Statistical comparisons demonstrating detectable cell surface expression (*, $p < 0.05$; ***, $p < 0.001$) are relative to the Δ444 mutant whose cell surface receptor expression was indistinguishable from the background HRP signal. Cell surface receptor levels for each truncated mutant GLP-2R were also compared with that of the WT GLP-2R (###, $p < 0.001$). *C*, BHK cells transiently transfected with either WT or mutant GLP-2Rs were treated with 0–100 nM GLP-2 for 10 min and assayed for total cAMP accumulation following treatment. Data (mean ± S.D., $n = 3$) were fit to sigmoidal dose-response curves using GraphPad Prism 3.03 and are representative of three independent experiments. *D*, BHK cells were transiently transfected with eight times less of WT than truncated mutant GLP-2R cDNAs to achieve similar levels of cell surface receptor expression as was confirmed by enzyme-linked immunosorbent assay. The ratio of maximal cAMP production (100 nM GLP-2, 10 min) to cell surface receptor expression was calculated for each receptor construct and expressed relative to the WT GLP-2R ratio. Data are mean ± S.D. ($n = 3$) and are representative of four independent experiments. Statistical comparisons are relative to the WT receptor ratio (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

tagged GLP-2Rs with progressively shorter C-terminal tails (Fig. 1). To assess the effects of C-terminal truncation on cell surface receptor expression and activation of second messenger signaling pathways, we transiently expressed either the FLAG-tagged wild-type (WT) or mutant GLP-2 receptors in baby hamster kidney fibroblasts (BHKs). Basal cell surface receptor expression was significantly decreased for all mutant receptors as assessed by an enzyme-linked immunosorbent assay (Fig. 2*A*). The mutant receptor lacking the entire predicted C terminus (Δ444) was not detected on the cell surface; however, six C-terminal amino acids (Δ450) or replacement of the C-terminal tail with five alanine residues (Δ4445A) was sufficient to restore detectable cell surface receptor expression (Fig. 2*A*, *inset*). The decrease in cell surface expression was not simply attributable to deficient cellular expression of the mutant receptor protein, because similar total cellular levels of WT and mutant GLP-2 receptor proteins were detected following cell permeabilization (Fig. 2*B*).

To determine the functional importance of the GLP-2R C-terminal sequences for signal transduction, we assessed GLP-2-stimulated cAMP accumulation for both wild type and mutant receptors. Surprisingly, cAMP accumulation following agonist stimulation of BHK cells transfected with the Δ533, Δ504, or Δ470 mutants was only modestly decreased compared with the WT receptor (Fig. 2*C*). The severely truncated Δ450 mutant exhibited a small, yet detectable GLP-2-induced cAMP response, whereas the Δ4445A and the Δ444 mutants did not activate adenylyl cyclase. Taken together with data in Fig. 2*A*, detectable cell surface expression is not dependent on the identity of amino acids in the proximal GLP-2R C terminus, but a small number of receptor-specific amino acids within this region are necessary for $G\alpha_s$ coupling. To directly compare the magnitude of receptor signaling, cells were transfected to express approximately equal levels of cell surface wild-type or mutant GLP-2Rs. When the maximal cAMP response for each mutant GLP-2R was expressed relative to the cell surface receptor density (Fig. 2*D*), progressive deletion of the GLP-2R C-terminal tail appears to paradoxically enhance receptor coupling to adenylyl cyclase. These observations suggest that, although the majority of the GLP-2R C terminus is dispensable

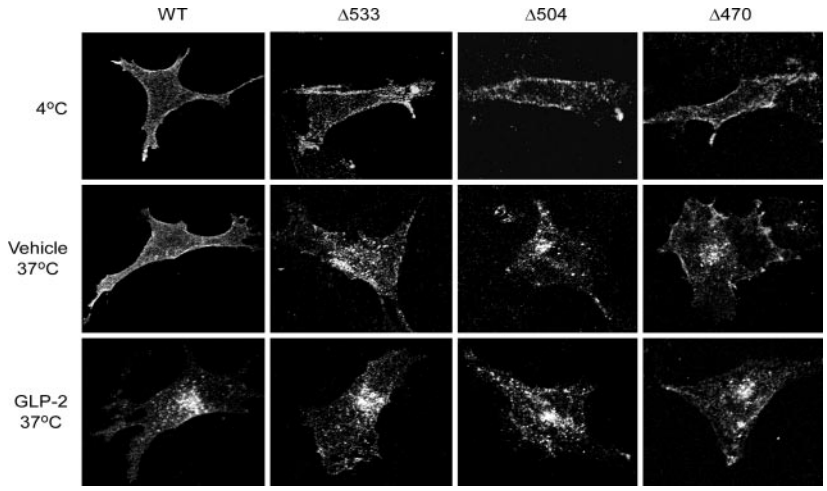

FIG. 3. **The C-terminal tail is required for retention of the GLP-2R at the plasma membrane, but not for agonist-induced internalization.** BHK cells transiently expressing either the WT or the indicated truncated mutant GLP-2 receptors were prelabeled with anti-FLAG (M1) antibody as described under "Experimental Procedures." Next, cells were incubated for 1 h either at 4 °C to prevent endocytosis, or at 37 °C in media alone (*Vehicle*) or with 10 nM GLP-2. Following fixation and permeabilization, the receptor/anti-FLAG antibody complexes were visualized by confocal microscopy using a Cy3-conjugated secondary antibody. Due to lower cell surface receptor levels for the truncated GLP-2R mutants, detector gain was increased to visualize labeled receptors. Images are representative of three independent experiments.

for activation of $G\alpha_s$, sequences within the distal region may act in part to constrain maximal levels of receptor signaling.

Truncation of the GLP-2R C Terminus Promotes Agonist-independent Receptor Endocytosis—The C-terminal tail of GPCRs may serve to ensure proper localization and/or stabilization of peptide hormone-activated GPCRs on the plasma membrane (18–22). To determine whether the decreased cell surface expression of mutant GLP-2Rs lacking portions of the C-terminal tail was due to differences in cellular localization and/or trafficking, BHK cells transiently transfected with either FLAG-tagged WT or mutant receptors were prelabeled with anti-FLAG antibody (M1), as described under "Experimental Procedures," and next incubated in media alone (vehicle) or with 10 nM GLP-2 for 60 min at 37 °C. Receptor-antibody complexes were visualized by confocal microscopy. Because intact cells were labeled with FLAG-antibody prior to stimulation, detected immunofluorescence represented receptor that was initially on the cell surface. Basal cell surface expression for the Δ450 and Δ4445A GLP-2R mutant receptors was difficult to monitor, hence subsequent experiments were performed using only the Δ533-, Δ504-, and Δ470-transfected GLP-2R receptor cDNAs.

When endocytosis was inhibited by incubation of the cells at 4 °C, the WT and the majority of the mutant-labeled receptors remained localized to the cell surface (Fig. 3, *top panels*). Following a 60-min incubation in media alone at 37 °C, the majority of the immunofluorescent signal associated with the prelabeled mutant receptors was detected within the cell, whereas the WT receptor remained predominantly associated with the plasma membrane (Fig. 3, *middle panels*). However, upon stimulation with GLP-2, all of the receptor constructs appeared to traffic to a similar perinuclear compartment within the cell (Fig. 3, *bottom panels*). No detectable immunofluorescence was observed when cells were transfected with either vector alone (pcDNA 3.1) or the Δ444 mutant (data not shown). Thus, truncation of the terminal 21 amino acids (Δ533 mutant) appears to promote constitutive GLP-2R internalization; however, elimination of the majority of the C terminus does not affect the ability of the receptor to undergo agonist-induced endocytosis and subsequent trafficking into the recycling compartment. Therefore, the distal GLP-2R C terminus may act to retain the un-liganded receptor on the plasma membrane.

The C-terminal Tail Regulates GLP-2R Trafficking following GLP-2-induced Receptor Endocytosis—Because GLP-2R internalization was not prevented by truncation of the C-terminal tail (Fig. 3), we addressed whether trafficking of the receptor back to the cell surface following endocytosis was altered by removal of C-terminal receptor sequences. We have recently characterized WT GLP-2R desensitization and trafficking using transfected human epithelial colon cancer (DLD-1) and BHK cell lines (17). Following treatment of either stably transfected DLD-1:GLP-2R (Fig. 4, *A* and *B*) or BHK:GLP-2R cells (data not shown) with vehicle or 10 nM GLP-2 for up to 20 min, agonist was removed by washing, and cell surface-associated receptor levels were measured following incubation in media alone for up to 2 h to monitor the kinetics of receptor reappearance. As shown previously (17), the WT GLP-2 receptor exhibited slow cell surface reappearance kinetics. However, the cell surface density of the truncated receptors recovered more rapidly than the WT receptor (Fig. 4*A*). In addition, the GLP-2R C-terminal-truncated mutants did not appear to exhibit enhanced receptor degradation, because the level of total cellular receptor protein for all transfected GLP-2 receptor cDNAs appeared comparable during the time course of treatment and recovery (Fig. 4*B*). Thus, truncation of the GLP-2R C terminus does not inhibit internalization or trafficking but allows a more rapid recovery of cell surface receptor levels following ligand-induced internalization.

The Distal 21 Amino Acids of the GLP-2R C Terminus Are Required for Agonist-induced Association with β-Arrestin-2—Many GPCRs associate with the non-visual arrestins, also referred to as the β-arrestins, following agonist stimulation (for review, see Ref. 23). Because the stability of the GPCR/β-arrestin interaction is suggested to predict the trafficking kinetics of receptors (24, 25), we addressed whether the GLP-2R interacts with β-arrestin-2. In BHK cells co-transfected with GFP-tagged β-arrestin-2 (β-arrestin-2-GFP) and either the WT or Δ470 mutant GLP-2Rs, β-arrestin-2-GFP was distributed throughout the cytoplasm in the un-stimulated state (Fig. 5, *A* and *B*, *middle panel*). Some co-localization of the WT receptor and β-arrestin-2-GFP was detected near the cell surface (Fig. 5*A*, *merge*). In contrast, the Δ470 mutant receptor was mostly detected within the intracellular space and did not associate with β-arrestin-2-GFP, in keeping with the constitutive internalization of this receptor (Fig. 5*B*; see also Fig. 3). Following exposure to 10 nM GLP-2 for 5, 20 (data not shown), or 60 min, extensive redistribution of β-arrestin-2-GFP was observed in the cells transfected with the WT receptor (Fig. 5*A*), but not the Δ470 mutant (Fig. 5*B*). Furthermore, the WT receptor internalized and remained associated with β-arrestin-2-GFP (Fig. 5*A*), whereas internalized Δ470 mutant GLP-2 receptors did not co-localize with β-arrestin-2-GFP (Fig. 5*B*).

To identify domains within the GLP-2 receptor C terminus responsible for interaction with β-arrestin-2, we performed similar experiments with the Δ504 (data not shown) and Δ533 mutant receptors. Consistent with findings using the

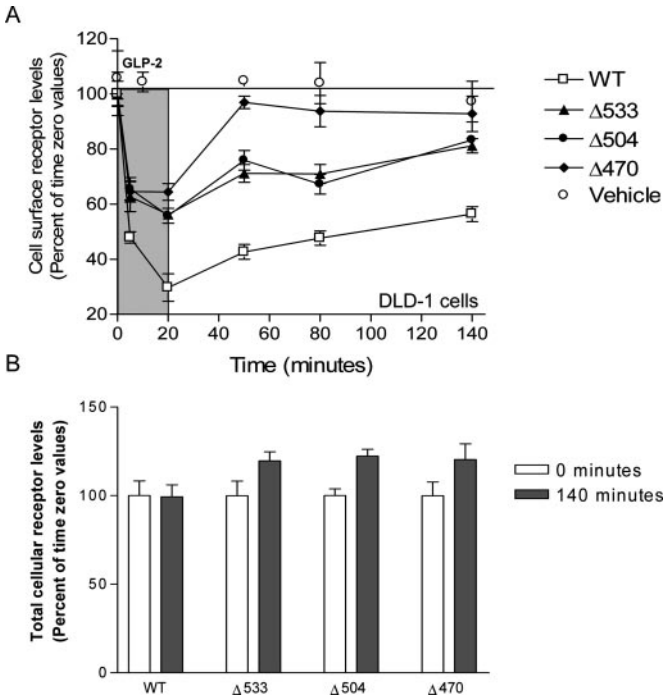

FIG. 4. **Truncation of the GLP-2R C-terminal tail alters agonist-induced receptor trafficking.** DLD-1 cells stably expressing either the FLAG-tagged WT or the indicated truncated mutant GLP-2 receptors were treated with media alone (*Vehicle*) or with 10 nM GLP-2 for 5–20 min to induce receptor internalization (*shaded area*). To monitor reappearance of receptors on the cell surface, cells were treated for 20 min with 10 nM GLP-2, washed, and then incubated for up to 120 min in the absence of agonist. Cell surface receptor levels (*A*) or total cellular receptor levels (*B*) at the indicated time points were measured using an HRP-linked immunosorbent assay, as described under "Experimental Procedures." Data for each receptor construct are expressed as a percentage of either cell surface receptor levels (*A*) or total cellular receptor levels (*B*) at time zero. Data points represent mean ± S.D. of triplicate values and are representative of two independent experiments.

WT GLP-2R, some co-localization of β-arrestin-2-GFP and the Δ533 mutant was detected near the plasma membrane in un-stimulated cells (Fig. 5*C*). However, neither redistribution of β-arrestin-2-GFP nor co-localization of β-arrestin-2-GFP with the Δ533 mutant GLP-2 receptor was observed following agonist treatment.

To address whether untagged β-arrestin-2 physically associated with the WT or mutant GLP-2 receptors in an agonist-dependent manner, cross-linking/immunoprecipitation experiments were performed in transfected BHK cells. Following a 60-min incubation with GLP-2, β-arrestin-2 was co-immunoprecipitated together with the FLAG-tagged WT GLP-2 receptor (Fig. 5*D*). In contrast, no detectable β-arrestin-2 was observed in immunoprecipitates of cells transfected with β-arrestin-2 and either the Δ533 or the Δ470 mutant (Fig. 5*D*). To address the possibility of a transient interaction between β-arrestin-2 and the Δ533 mutant receptor, cross-linking and immunoprecipitation was also performed following a 5-, 20-, and 60-min treatment with GLP-2. Although the association of β-arrestin-2 with the WT GLP-2 receptor increased in a time-dependent manner, with maximal levels detected after a 60-min incubation with agonist (Fig. 5*E*), no β-arrestin-2 was detected in immunoprecipitates of the Δ533 mutant at any time point (Fig. 5*F*). Taken together, this data suggest that the distal 21 amino acids of the GLP-2R C-terminal tail are necessary for agonist-induced, stable binding of β-arrestin-2. Furthermore, although β-arrestin-2 appeared to extensively associate and internalize with the WT GLP-2 receptor, this association was not required for ligand-induced endocytosis or intracellular trafficking, because these processes were preserved for mutant GLP-2 receptors that do not bind β-arrestin-2 (Figs. 3, 4*A*, 5*B*, and 5*C*).

Mutation of Putative Phosphorylation Sites within the Distal GLP-2R C Terminus Disrupts Agonist-induced Association with β-Arrestin-2—Phosphorylation of specific amino acid sequences within intracellular receptor domains by G-protein receptor kinases is believed to direct β-arrestin binding to GPCRs (for review, see Ref. 12). To identify specific GLP-2R residues that regulate the β-arrestin-2-receptor interaction, we eliminated putative phosphorylation sites by mutating specific serine residues to alanine within the C terminus (Fig. 6*A*). The single mutation of Ser-551 to alanine (S551A) did not appear to diminish β-arrestin-2 binding following agonist stimulation as assessed following immunoprecipitation of the FLAG-tagged mutant receptor from transfected BHK cells (Fig. 6, *B* and *C*). Although a GLP-2 receptor that had the serine cluster Ser-528/Ser-529/Ser-531 mutated (AALA) exhibited an approximate 50% decreased association with β-arrestin-2, a receptor containing both the S551A and AALA mutations exhibited an even greater reduction in β-arrestin-2 association (25% of WT) following stimulation with agonist for 60 min (Fig. 6, *B* and *C*). The decreased, but not completely abrogated, association of the mutated GLP-2Rs with β-arrestin-2 is similar to findings previously reported for the neurotensin-1 receptor, oxytocin receptor, angiotensin II type 1A receptor, and substance P receptors (26). Mutation of two additional upstream serine residues (S460A and S482A) did not further decrease the association with β-arrestin-2. Taken together, these data provide additional evidence that the distal C terminus directs agonist-induced β-arrestin-2 binding and suggest that this stable association most likely occurs following phosphorylation of specific serine residues within this domain.

The GLP-2R C-terminal Tail Is Dispensable for Ligand-induced Receptor Desensitization and Subsequent Resensitization, but Is Required for PKA-mediated Down-regulation of GLP-2R Signaling—Because β-arrestin is known to play a key role in the desensitization of many GPCRs, most likely through its ability to uncouple the receptors from G-protein subunits, we addressed whether mutant receptors lacking the ability to bind β-arrestin-2 could undergo homologous desensitization. Because the S551A/AALA mutant receptor exhibited low, but detectable, binding to β-arrestin-2, we chose to examine GLP-2R desensitization and resensitization kinetics using the C-terminal truncated mutants, whose association with β-arrestin-2 was completely eliminated. Stably transfected DLD-1 cells expressing either WT or truncated mutant GLP-2 receptors were pre-treated with either vehicle (control) or 10 nM GLP-2, washed, and re-incubated in media alone for indicated periods of time (recovery) prior to re-challenge with 100 nM GLP-2 to assess the extent of agonist-induced desensitization (Fig. 7). Surprisingly, all of the truncated receptors underwent rapid, GLP-2-induced (homologous) desensitization followed by slow resensitization kinetics similar to the WT receptor. Therefore, it does not appear that regulatory domains within the GLP-2R C terminus, including the amino acids necessary for β-arrestin-2 binding, are required for desensitization of receptor signaling or the subsequent recovery of receptor responsiveness. Moreover, we do not see a correlation between the kinetics of receptor resensitization (Fig. 7) and receptor reappearance on the cell surface (Fig. 4*A*), which is similar to our previously published findings (17) and suggests that recovery of basal cell surface GLP-2R levels may not be sufficient for recovery of receptor signaling following agonist desensitization.

β-Arrestin-2 binding does not appear necessary for homologous GLP-2R desensitization; however, phosphorylation of in-

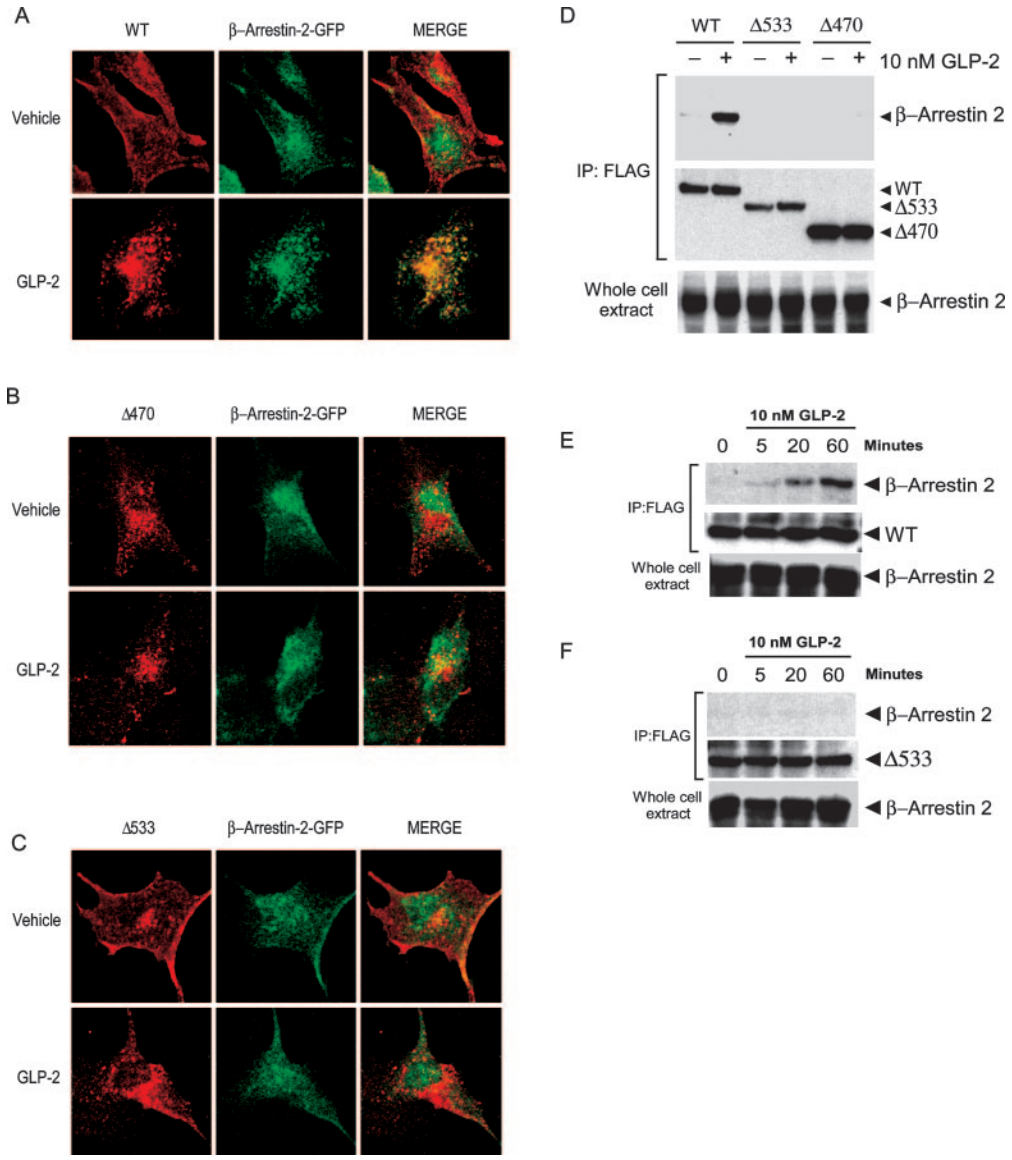

FIG. 5. **The GLP-2R distal C terminus is required for agonist-induced association with β-arrestin-2.** *A–C*, BHK cells transiently transfected with β-arrestin-2-GFP and either WT (*A*), Δ470 (*B*), or Δ533 (*C*) truncated mutant GLP-2 receptors were prelabeled with anti-FLAG (M1) antibody as described under "Experimental Procedures." Cells were then incubated for 60 min at 37 °C in media alone (*Vehicle*) or with 10 nM GLP-2. Following fixation and permeabilization, the receptor/anti-FLAG antibody complexes were visualized by confocal microscopy using a Cy3-conjugated secondary antibody. *D–F*, BHK cells were transiently transfected with untagged β-arrestin-2 and either WT or the indicated truncated mutant GLP-2 receptor constructs. Following incubation in the absence (−) or presence (+) of 10 nM GLP-2 for 60 min (*D*) or for the indicated times (*E* and *F*), cells were cross-linked, harvested, and extracted as described under "Experimental Procedures." Equal amounts of protein from each cleared cell lysate were immunoprecipitated with anti-FLAG (M1) antibody and subjected to Western blot analysis to detect co-immunoprecipitated β-arrestin-2 or the FLAG-tagged GLP-2 receptor constructs. Similar β-arrestin-2 expression levels between transfections were confirmed by Western blot analysis of whole cell extracts. Images and blots are representative of two to three independent experiments.

tracellular domains by second-messenger kinases can also facilitate down-regulation of GPCR signaling (heterologous desensitization) independently of arrestin. To address whether this mechanism was responsible for GLP-2R desensitization, we treated DLD-1 (Fig. 8) or BHK cells (data not shown) stably expressing the wild-type GLP-2R with activators of either protein kinase A (PKA) or protein kinase C (PKC). Although activation of PKC with 400 nM phorbol 12-myristate 13-acetate did not result in down-regulation of GLP-2R signaling (data not shown), pre-treatment of cells with 20 μM forskolin (FK), a specific activator of adenylyl cyclase, decreased agonist-stimulated cAMP accumulation to a similar extent as pre-treatment with GLP-2 (Fig. 8*A*). Moreover, concurrent pre-treatment of cells with both GLP-2 and FK further potentiated GLP-2R desensitization. Surprisingly, although inhibition of PKA activity with 10 μM H-89 blocked FK-induced GLP-2R desensitization, it had no significant effect on ligand-induced desensitization (Fig. 8*B*). Consistent with a role of the GLP-2R C terminus in heterologous, but not homologous desensitization, progressive truncation of this domain attenuated FK-, but not agonist-mediated GLP-2R desensitization (Fig. 8*C*). Therefore, although PKA activation can regulate GLP-2R signaling via the C-terminal domain, neither PKA activity nor β-arrestin-2 binding appear necessary for down-regulation of GLP-2R signaling in response to ligand. This suggests the existence of a novel, currently uncharacterized, mechanism for homologous GLP-2R desensitization.

Ligand-induced GLP-2R ERK 1/2 Activation Requires Coupling to $G\alpha_{i/o}$, but Is β-Arrestin-2- and PKC-independent—GPCRs can activate MAPK signaling pathways through the recruitment of β-arrestin-2 (for review see Refs. 23 and 27). Previous studies of GLP-2R signaling in BHK cells did not

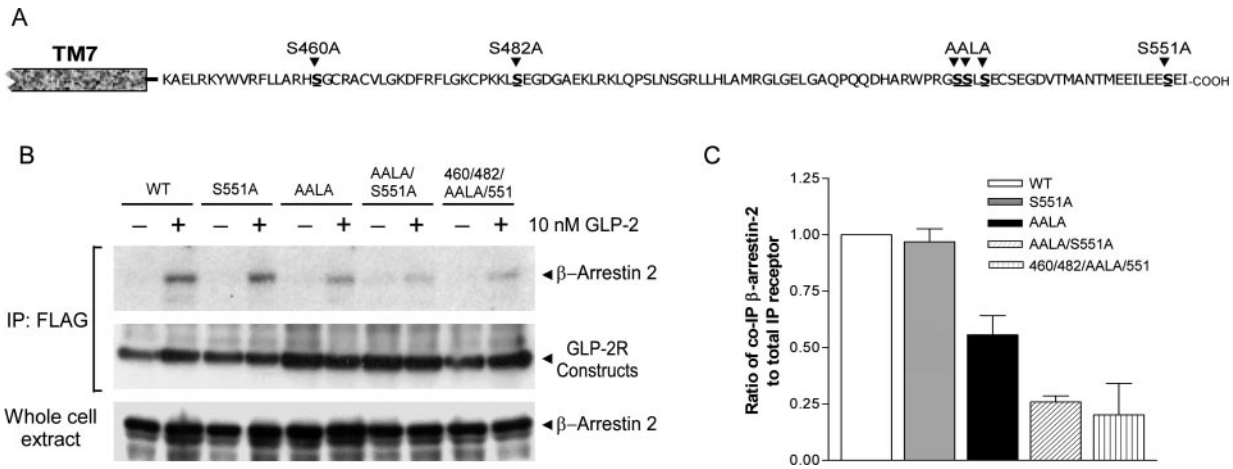

FIG. 6. **Mutation of putative phosphorylation sites within the distal GLP-2R C-terminal tail disrupt ligand-induced β-arrestin-2 recruitment.** *A*, schematic representation of the GLP-2R C-terminal amino acid sequence downstream of the predicted 7th transmembrane domain (*TM7*). Individual or clusters of serine residues (*underlined* and *highlighted*) were mutated to alanine by site-directed mutagenesis. *B*, BHK cells transiently expressing either WT or mutant GLP-2 receptors possessing single or multiple point mutations as indicated, were incubated in the absence (−) or presence (+) of 10 nM GLP-2 for 60 min prior to cross-linking, anti-FLAG(M1) antibody immunoprecipitation (*IP*) and Western blot analysis as described in Fig. 5. Similar β-arrestin-2 expression levels between transfections were confirmed by Western blot analysis of whole cell extracts. Blots are representative of two independent experiments. The ratios of co-immunoprecipitated β-arrestin-2 to total immunoprecipitated receptor, calculated for each receptor mutant construct and expressed relative to the WT GLP-2R ratio, are shown in *C*. Quantification was performed by densitometry. Data are mean ± range of two independent experiments.

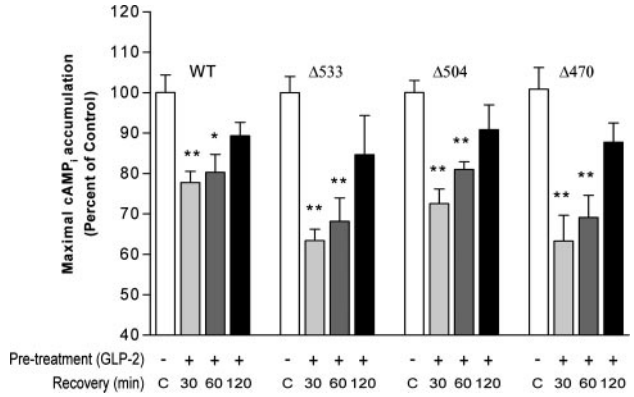

FIG. 7. **The C-terminal tail of the GLP-2R is not required for homologous receptor desensitization or resensitization.** DLD-1 cells stably expressing either the WT or the indicated truncated mutant GLP-2 receptors were incubated for 20 min in the absence (−) or presence (+) of 10 nM GLP-2 (pre-treatment), washed, and allowed to recover for either 30, 60, or 120 min. Cells were then re-challenged with 100 nM GLP-2 for 10 min prior to measuring intracellular cAMP content. Data for each receptor construct (mean ± S.E. from two independent experiments each performed in quadruplicate) are expressed as a percentage of their corresponding control (*c*) (pre-treatment in the absence of GLP-2 and additional 120-min recovery). Statistical comparisons are relative to the control (**, $p < 0.01$; ***, $p < 0.001$).

detect significant ERK1/2 phosphorylation following agonist treatment (28). However, GLP-2 treatment of HeLa cells transfected with the GLP-2R led to activation of ERK1/2 that required both $G\alpha_{i/o}$ and $\beta\gamma$ subunits, was Ras-dependent, and was not the result of epidermal growth factor receptor transactivation (8). Therefore, we used HeLa cells transfected with either the WT or mutant GLP-2 receptors to address whether GLP-2R-mediated activation of ERK1/2 was dependent on receptor association with β-arrestin-2. Similar patterns of β-arrestin-2 association to the WT and Δ533 mutant receptors, as assessed by cross-linking/immunoprecipitation, were observed in HeLa and BHK cells (data not shown). Increased ERK1/2 phosphorylation was observed following short-term GLP-2 treatment of HeLa cells transfected with either the WT, Δ533, Δ504, Δ470, or 460/482/AALA/551 mutant GLP-2 receptors, but not the Δ450, Δ4445A, or Δ444 mutants (Fig. 9*A*). Therefore, elimination of the majority of the GLP-2R C terminus or mutations, which attenuate β-arrestin-2 association, do not prevent activation of the ERK1/2 MAPK signaling pathway.

It was recently suggested that early onset and transient GPCR-mediated ERK1/2 phosphorylation is dependent on G-protein-regulated PKC activity, whereas delayed onset and persistent ERK1/2 activation is dependent on β-arrestin-2 (29). Both the WT and Δ533 mutant receptors exhibited identical time courses of rapid and transient ERK1/2 phosphorylation following GLP-2 treatment in HeLa cells (Fig. 9*B*), consistent with our data suggesting that GLP-2R MAPK activation is β-arrestin-2-independent. The transient increase in ERK1/2 phosphorylation following acute stimulation of both the WT and Δ533 GLP-2 receptor mutants was abrogated by pertussis toxin pre-treatment (Fig. 9*C*) but was not affected following inhibition of PKC with Ro-31-8425 (Fig. 9*B*), although the PKC inhibitor efficiently blocked phorbol 12-myristate 13-acetate-induced ERK1/2 phosphorylation (Fig. 9*B*). These data demonstrate that GLP-2R-mediated ERK1/2 phosphorylation is G-protein-dependent but does not require PKC activation or the C-terminal domain of the receptor, including amino acid residues necessary for interaction with β-arrestin-2.

DISCUSSION

In this study we show that the progressive truncation of the predicted GLP-2 receptor C-terminal tail did not abrogate agonist-induced, G-protein-mediated activation of the adenylyl cyclase or MAPK signaling pathways. However, the C terminus was required for proper cell surface receptor expression, yet not for ligand-induced endocytosis, because truncated mutant receptors were rapidly internalized and targeted to a conserved intracellular compartment following incubation with GLP-2. Following endocytosis, truncated mutant receptors reappeared on the cell surface more rapidly than the full-length receptor and failed to stably associate with β-arrestin-2. Mutation of putative phosphorylation sites within the distal GLP-2R C terminus diminished ligand-induced β-arrestin-2 association. However, disruption of stable, ligand-induced β-arrestin-2 binding to the GLP-2R, by site-directed mutagenesis or truncation of the GLP-2R, did not affect transient, PKC-independent ERK1/2 phosphorylation.

Truncation of the distal GLP-2R C terminus appears to

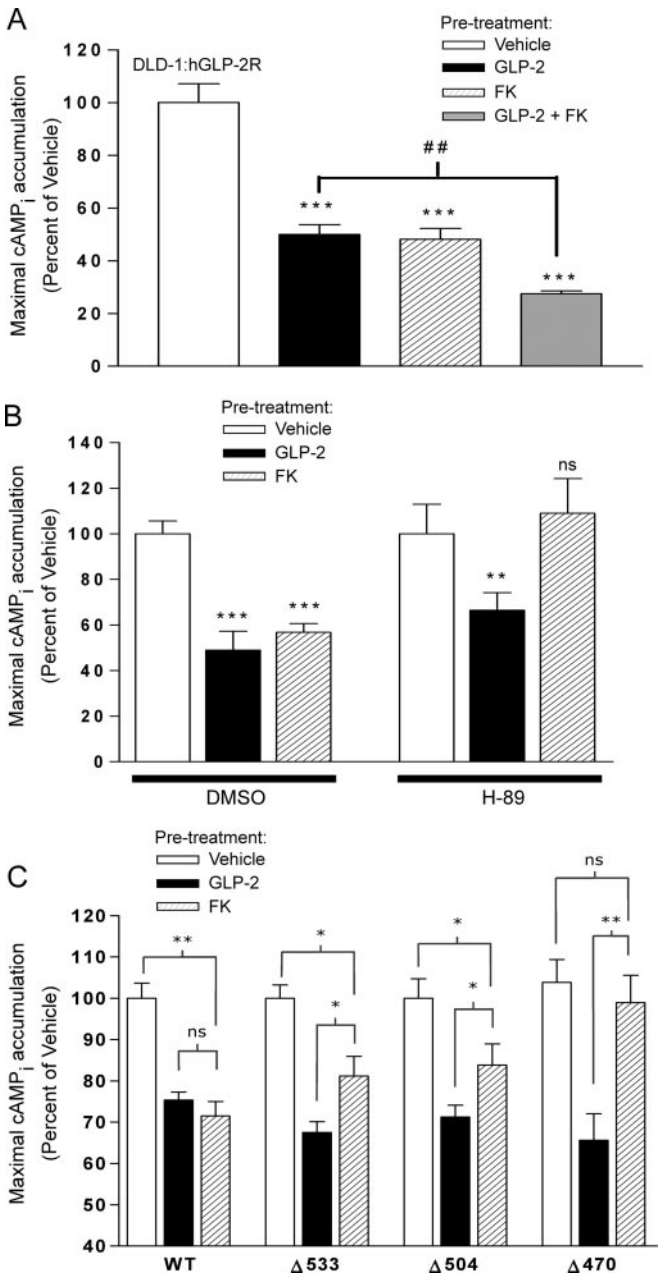

FIG. 8. **PKA-mediated heterologous GLP-2R desensitization requires domains within the receptor C terminus.** *A*, DLD-1:hGLP-2R cells were pre-treated for 20 min with either vehicle (media alone), 10 nM GLP-2, 20 μM FK, or both GLP-2 and FK, prior to a 30-min recovery period and subsequent assessment of GLP-2-induced intracellular cAMP accumulation as described in Fig. 7. *B*, DLD-1:hGLP-2R cells were incubated in media containing either vehicle (Me_2SO (*DMSO*)) or 10 μM H-89 to inhibit PKA activity prior to, during desensitization, and throughout recovery as in *A*. Data (mean ± S.D.) are expressed as a percentage of their corresponding control (vehicle pre-treatment) and are representative of a least three independent experiments performed in triplicate. Statistical comparisons are relative to the vehicle control (*white bars*), and $^{\#\#}$, $p < 0.01$ for GLP-2 *versus* GLP-2 plus FK. *C*, desensitization of the WT or truncated mutant GLP-2Rs in response to either 10 nM GLP-2 or 20 μM FK was assessed in stably transfected DLD-1 cells. Data for each receptor construct (mean ± S.E. from two independent experiments each performed in quadruplicate) are expressed as a percentage of their corresponding control (*Vehicle*). Statistical comparisons in *A–C*, as indicated, are *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and *ns*, not significant.

promote constitutive internalization while not preventing G-protein-coupled signaling, thus suggesting that the C terminus directs stabilization of un-stimulated GLP-2Rs at the plasma membrane. This characteristic may be conserved among hormone-activated GPCRs, because many receptors with peptide ligands (18, 19, 21, 22) exhibit decreased cell surface expression following truncation of this intracellular domain. Moreover, C-terminal truncation of the somatostatin receptor 14 similarly promotes agonist-independent internalization (30). This suggests the existence of as yet unidentified conserved C-terminal structural motifs that direct proper membrane retention of GPCRs activated by peptides. Surprisingly, progressive truncation of the C terminus enhanced the ability of the GLP-2R to activate adenylyl cyclase, an observation also reported for the highly related glucagon receptor (31) suggesting that this domain may tonically repress $G\alpha_s$ signaling. Our data demonstrate that the GLP-2R C terminus is not necessary for G-protein-dependent effector activation but is required for proper expression and/or retention of the receptor at the plasma membrane.

Recently, GPCRs have been further classified into two subgroups based on their recycling kinetics and the specificity and stability of β-arrestin binding. "Class A" GPCRs interact differentially and transiently with the arrestin isoforms and tend to recycle quickly, whereas "Class B" GPCRs form a stable interaction with arrestin and traffic more slowly through the cell (24, 25, 32). Our current data, along with previously published findings (17), suggests that the wild-type GLP-2R, like the vasopressin-2, angiotensin II type 1A, thyrotropin-releasing hormone, neurokinin-1, and neurotensin-1 receptors, behaves as a Class B GPCR, based on the delayed trafficking kinetics and the prolonged interaction between the receptor and β-arrestin-2 (25). Furthermore, reduction of the ligand-induced β-arrestin-2 association by mutation of serine residues within the distal GLP-2R C terminus suggests that phosphorylation of this domain regulates stable binding of β-arrestin-2, consistent with a Class B GPCR (26). In contrast, it is tempting to propose that the truncated mutant receptors exhibit more Class A-like characteristics based on their recovery kinetics. However, the faster recovery of cell surface-truncated GLP-2R levels may be due to more rapid trafficking of nascent or constitutively internalized receptors from intracellular pools. Furthermore, because the C-terminal truncated mutant receptors did not appear to interact with β-arrestin-2, they do not conform to the classic definition of a Class A GPCR. Nevertheless, because we have only assessed the β-arrestin-2 interaction at fixed time points following ligand stimulation, we cannot exclude the possibility that the truncated mutant receptors transiently interact with β-arrestin-2.

The β-arrestins control a diverse array of functions in the regulation of GPCR signaling, including the following: potentiation of GPCR desensitization by uncoupling receptors from G-proteins; facilitation of receptor endocytosis; and serving as a scaffold to link GPCRs with intracellular signaling machinery (for review, see Ref. 33). One may argue that the enhanced cAMP accumulation observed for the truncated mutant receptors may in fact represent defective desensitization, thus implying a role for the C terminus and/or β-arrestin-2 association in the down-regulation of GLP-2R signaling. However, our results indicated that a truncated GLP-2R missing most of the C terminus (Δ470 mutant), including the putative β-arrestin-2 binding domain and the majority of available phosphorylation sites, underwent rapid, homologous desensitization to a similar extent as wild-type receptor. Interestingly, elimination of the C-terminal tail does abrogate PKA-mediated heterologous GLP-2R desensitization. Therefore, it is possible that the enhanced basal signaling of the truncated mutants is the result of deficient regulation by agonist-independent PKA activity. Resensitization of the GLP-2R following acute agonist stimulation requires reappearance of receptors back on the cell surface

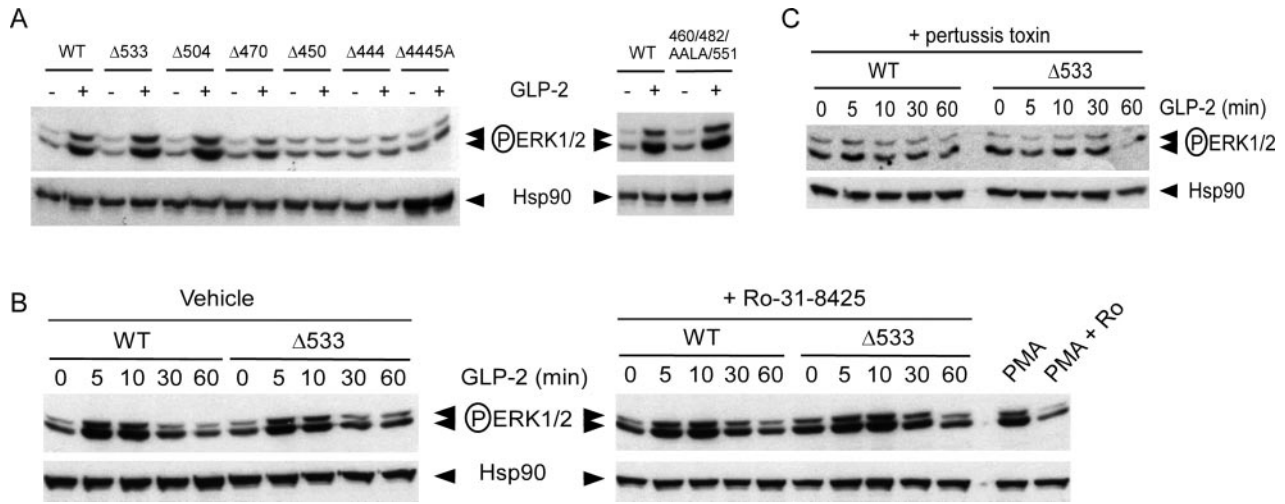

FIG. 9. **GLP-2R-dependent ERK1/2 activation in HeLa cells does not involve PKC or β-arrestin-2, but is mediated through $G\alpha_{i/o}$.** *A*, HeLa cells were transiently transfected with 10 times less of the WT and 460/482/AALA/551 mutant GLP-2R cDNAs than of the truncated mutant receptor cDNAs, to achieve similar levels of cell surface receptor expression (confirmed by enzyme-linked immunosorbent assay). 24 h following transfection, cells were serum-starved overnight and then stimulated with 20 nM GLP-2 for 5 min. Levels of phosphorylated ERK1/2, or Hsp90 as a loading control, were assessed by Western blot analysis of whole cell lysates. Blots are representative of four independent experiments. *B*, WT or Δ533 mutant GLP-2R-transfected HeLa cells were serum-starved overnight and then pre-treated with vehicle alone or 1 μM Ro-31-8425 for 1 h prior to stimulation with 20 nM GLP-2 for the indicated periods of time. To monitor the effectiveness of the PKC inhibitor, cells were pretreated with or without Ro-31-8425 prior to stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA) for 5 min. *C*, HeLa cells, transfected with either WT or the Δ533 mutant GLP-2Rs, were pre-treated with 200 ng/ml pertussis toxin for 24 h, serum-starved overnight, and then stimulated with 20 nM GLP-2 for the indicated periods of time. For *B* and *C*, whole cell lysates were immunoblotted as in *A* and are representative blots from three independent experiments.

following internalization (17), a regulatory mechanism shared by many GPCRs (12). However, consistent with our previous report, our results illustrating the differential trafficking, yet similar resensitization kinetics, of the wild-type and truncated receptors further demonstrate that restoration of the basal cell surface GLP-2R receptor levels is not sufficient for recovery of receptor responsiveness. Thus, we conclude that the mechanisms regulating homologous desensitization, and subsequent resensitization, of the GLP-2R occur independently of phosphorylation or binding of β-arrestin and/or other C-terminal tail-associated proteins. We are currently attempting to identify the unique mechanism governing ligand-induced reductions in GLP-2 signaling, which may involve phosphorylation-independent G-protein receptor kinase activity (34, 35) or activation of the regulators of G-protein signaling proteins (see Ref. 36 for review).

Interaction with the β-arrestins is also known to direct GPCR endocytosis in clathrin-coated pits via interaction of β-arrestin with the clathrin heavy chain and adapter protein comlex AP-2 (37, 38). Truncation of receptors or elimination of arrestin binding has been shown to prevent endocytosis of GPCRs utilizing this internalization pathway (32, 39, 40). Consistent with previously published data demonstrating that GLP-2R endocytosis occurs via a clathrin- and dynamin-independent, lipid-raft-dependent mechanism (17), the disruption of the GLP-2R/β-arrestin-2 interaction does not inhibit GLP-2R endocytosis. Nevertheless, we show that truncation of the GLP-2R C terminus does appear to modulate intracellular receptor trafficking following ligand-induced internalization, suggesting that the interaction with arrestin may regulate intracellular trafficking downstream of endocytosis. Although we have previously characterized the clathrin- and dynamin-independent mechanism of GLP-2R endocytosis in multiple cell lines heterologously expressing varying levels of receptor protein (17),[2] we cannot exclude the possibility that the GLP-2R may utilize alternative internalization pathways depending on the level of surface expression or the cellular context. Identification of a cell line that expresses the endogenous GLP-2R would facilitate further characterization of the mechanisms regulating GLP-2R trafficking.

Because a stable interaction between β-arrestin-2 and the GLP-2R does not appear to mediate homologous desensitization or receptor internalization, we hypothesized that β-arrestin-2 recruitment may link GLP-2R signaling to G-protein-independent effector pathways, namely ERK1/2 activation. The β-arrestins can facilitate Ras-mediated MAPK activation via interaction with Src (41), or by regulating transactivation of receptor tyrosine kinases (27, 42). Furthermore, the β-arrestins can recruit and scaffold ERK1/2, JNK3, and their upstream kinases (43–45), mediating a direct interaction between GPCRs and the components of two MAPK signaling pathways. However, disruption of the interaction between GLP-2R and β-arrestin-2, either by GLP-2R C-terminal truncation or site-directed mutagenesis, did not abrogate GLP-2-induced ERK1/2 phosphorylation. The transient time course of ERK1/2 phosphorylation following activation of either the wild-type or mutant GLP-2Rs is consistent with this observation, because signaling via β-arrestin-2 promotes delayed onset and sustained MAPK activation (29, 46). Moreover, inhibition of $G\alpha_{i/o}$-mediated signaling abrogates GLP-2-induced ERK1/2 phosphorylation by both wild-type and mutant receptors, further confirming that GLP-2R MAPK activation is G-protein-dependent.

Interestingly, inhibition of PKC did not block GLP-2-induced ERK1/2 phosphorylation in transfected cells, although PKC has been implicated in the regulation of β-arrestin-2-independent MAPK activation by GPCRs (46, 47). In general, PKC is believed to act downstream of $G\alpha_q$-mediated PLC activation; however, GPCR-mediated MAPK signaling via $G\alpha_{i/o}$ has been previously reported (48), where it was suggested to facilitate receptor tyrosine kinase transactivation. Although activation of the GLP-2R in HeLa cells does not result in epidermal growth factor receptor transactivation

[2] J. L. Estall, J. A. Koehler, B. Yusta, and D. J. Drucker, unpublished results.

(8), it remains possible that the GLP-2R modulates signaling of other receptor tyrosine kinases. Nevertheless, delineation of the PKC/β-arrestin-2-independent mechanism linking the GLP-2R, and possibly other GPCRs, to the MAPK signaling cascade is of substantial interest.

Because many GPCRs associate with both β-arrestin-1 and β-arrestin-2 (25), the truncated mutant GLP-2 receptors may retain the ability to interact with β-arrestin-1. Although it is possible that interaction with this arrestin isoform directs GLP-2 receptor desensitization, resensitization, and coupling to MAPK, preliminary data from our laboratory suggests that the GLP-2R does not physically associate with β-arrestin-1.[3] Accordingly, we conclude that disruption of the GLP-2R-β-arrestin interaction does not prevent GLP-2R desensitization, internalization, or G-protein-dependent effector activation. Thus, the functional relevance of the agonist-dependent stable β-arrestin-2 association with the GLP-2R has yet to be determined and warrants further investigation.

In summary, our data demonstrate that the C terminus directs proper plasma membrane GLP-2 receptor expression and stabilization, stable recruitment of β-arrestin-2, and ligand-induced receptor sequestration within the cell. Thus, the C terminus appears to somehow link the human GLP-2R receptor to intracellular sorting machinery. Taken together with our previously published observations (17), these results suggest that the mechanisms regulating GLP-2R desensitization, resensitization, and coupling to G-protein-dependent effector activation are distinct from those previously characterized for many members of the GPCR superfamily. Furthermore, our study suggests that β-arrestin-2 may possess a currently unidentified function in the regulation of GPCR activity unrelated to desensitization, endocytosis, or coupling to MAPK activation.

Acknowledgments—We thank Dr. M. G. Caron and Dr. S. Ferguson for generously providing the expression constructs for β-arrestin-2-GFP and wild-type β-arrestin-2, respectively.

REFERENCES

1. Drucker, D. J. (2001) *Endocrinology* **142,** 521–527
2. Munroe, D. G., Gupta, A. K., Kooshesh, F., Vyas, T. B., Rizkalla, G., Wang, H., Demchyshyn, L., Yang, Z. J., Kamboj, R. K., Chen, H., McCallum, K., Sumner-Smith, M., Drucker, D. J., and Crivici, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1569–1573
3. Yusta, B., Huang, L., Munroe, D., Wolff, G., Fantaske, R., Sharma, S., Demchyshyn, L., Asa, S. L., and Drucker, D. J. (2000) *Gastroenterology* **119,** 744–755
4. Bjerknes, M., and Cheng, H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12497–12502
5. Orskov, C., Hartmann, B., Poulsen, S. S., Thulesen, J., Hare, K. J., and Holst, J. J. (2005) *Regul. Pept.* **124,** 105–112
6. Yusta, B., Boushey, R. P., and Drucker, D. J. (2000) *J. Biol. Chem.* **275,** 35345–35352
7. Yusta, B., Estall, J., and Drucker, D. J. (2002) *J. Biol. Chem.* **277,** 24896–24906
8. Koehler, J. A., Yusta, B., and Drucker, D. J. (2005) *Mol. Endocrinol.* **19,** 459–473
9. Drucker, D. J., Erlich, P., Asa, S. L., and Brubaker, P. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7911–7916
10. Tsai, C. H., Hill, M., Asa, S. L., Brubaker, P. L., and Drucker, D. J. (1997) *Am. J. Physiol.* **273,** E77–E84
11. Estall, J. L., and Drucker, D. J. (2003) *J. Nutr.* **133,** 3708–3711
12. Ferguson, S. S. (2001) *Pharmacol. Rev.* **53,** 1–24
13. Mayo, K. E., Miller, L. J., Bataille, D., Dalle, S., Goke, B., Thorens, B., and Drucker, D. J. (2003) *Pharmacol. Rev.* **55,** 167–194
14. Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G., and Shetzline, M. A. (1999) *J. Biol. Chem.* **274,** 31515–31523
15. Chauvin, S., Bencsik, M., Bambino, T., and Nissenson, R. A. (2002) *Mol. Endocrinol.* **16,** 2720–2732
16. Rasmussen, T. N., Novak, I., and Nielsen, S. M. (2004) *Eur. J. Biochem.* **271,** 4366–4374
17. Estall, J. L., Yusta, B., and Drucker, D. J. (2004) *Mol. Biol. Cell* **15,** 3673–3687
18. Iida-Klein, A., Guo, J., Xie, L. Y., Juppner, H., Potts, J. T., Jr., Kronenberg, H. M., Bringhurst, F. R., Abou-Samra, A. B., and Segre, G. V. (1995) *J. Biol. Chem.* **270,** 8458–8465
19. Buggy, J. J., Heurich, R. O., MacDougall, M., Kelley, K. A., Livingston, J. N., Yoo-Warren, H., and Rossomando, A. J. (1997) *Diabetes* **46,** 1400–1405
20. Lin, X., Janovick, J. A., Brothers, S., Blomenrohr, M., Bogerd, J., and Conn, P. M. (1998) *Mol. Endocrinol.* **12,** 161–171
21. Blomenrohr, M., Heding, A., Sellar, R., Leurs, R., Bogerd, J., Eidne, K. A., and Willars, G. B. (1999) *Mol. Pharmacol.* **56,** 1229–1237
22. Wheeler, M. B., Gelling, R. W., Hinke, S. A., Tu, B., Pederson, R. A., Lynn, F., Ehses, J., and McIntosh, C. H. (1999) *J. Biol. Chem.* **274,** 24593–24601
23. Pierce, K. L., and Lefkowitz, R. J. (2001) *Nat. Rev. Neurosci.* **2,** 727–733
24. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *J. Biol. Chem.* **274,** 32248–32257
25. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) *J. Biol. Chem.* **275,** 17201–17210
26. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (2001) *J. Biol. Chem.* **276,** 19452–19460
27. Pierce, K. L., Luttrell, L. M., and Lefkowitz, R. J. (2001) *Oncogene* **20,** 1532–1539
28. Yusta, B., Somwar, R., Wang, F., Munroe, D., Grinstein, S., Klip, A., and Drucker, D. J. (1999) *J. Biol. Chem.* **274,** 30459–30467
29. Ahn, S., Shenoy, S. K., Wei, H., and Lefkowitz, R. J. (2004) *J. Biol. Chem.* **279,** 35518–35525
30. Schwartkop, C. P., Kreienkamp, H. J., and Richter, D. (1999) *J. Neurochem.* **72,** 1275–1282
31. Unson, C. G., Cypess, A. M., Kim, H. N., Goldsmith, P. K., Carruthers, C. J., Merrifield, R. B., and Sakmar, T. P. (1995) *J. Biol. Chem.* **270,** 27720–27727
32. Shenoy, S. K., and Lefkowitz, R. J. (2003) *Biochem. J.* **375,** 503–515
33. Luttrell, L. M., and Lefkowitz, R. J. (2002) *J. Cell Sci.* **115,** 455–465
34. Dhami, G. K., Anborgh, P. H., Dale, L. B., Sterne-Marr, R., and Ferguson, S. S. (2002) *J. Biol. Chem.* **277,** 25266–25272
35. Dhami, G. K., Dale, L. B., Anborgh, P. H., O'Connor-Halligan, K. E., Sterne-Marr, R., and Ferguson, S. S. (2004) *J. Biol. Chem.* **279,** 16614–16620
36. Zhong, H., and Neubig, R. R. (2001) *J. Pharmacol. Exp. Ther.* **297,** 837–845
37. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383,** 447–450
38. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J. Biol. Chem.* **275,** 23120–23126
39. Parent, J. L., Labrecque, P., Orsini, M. J., and Benovic, J. L. (1999) *J. Biol. Chem.* **274,** 8941–8948
40. Qian, H., Pipolo, L., and Thomas, W. G. (2001) *Mol. Endocrinol.* **15,** 1706–1719
41. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) *Science* **283,** 655–661
42. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402,** 884–888
43. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) *J. Cell Biol.* **148,** 1267–1281
44. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2449–2454
45. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) *Science* **290,** 1574–1577
46. Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., and Lefkowitz, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10782–10787
47. Wei, H., Ahn, S., Barnes, W. G., and Lefkowitz, R. J. (2004) *J. Biol. Chem.* **279,** 48255–48261
48. Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1489–1494

[3] J. L. Estall, J. A. Koehler, B. Yusta, and D. J. Drucker, unpublished observations.