Phosphorylation of β-Arrestin2 Regulates Its Function in Internalization of β_2-Adrenergic Receptors†

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ABSTRACT: β-Arrestins mediate agonist-dependent desensitization and internalization of G protein-coupled receptors. Previously, we have shown that phosphorylation of β-arrestin1 by ERKs at Ser-412 regulates its association with clathrin and its function in promoting clathrin-mediated internalization of the receptor. In this paper we report that β-arrestin2 is also phosphorylated, predominantly at residues Thr-383 and Ser-361. Isoproterenol stimulation of the β_2-adrenergic receptor promotes dephosphorylation of β-arrestin2. Mutation of β-arrestin2 phosphorylation sites to aspartic acid decreases the association of β-arrestin2 with clathrin, thereby reducing its ability to promote internalization of the β_2-adrenergic receptor. Its ability to bind and desensitize the β_2-adrenergic receptor is, however, unaltered. These results suggest that, analogous to β-arrestin1, phosphorylation/dephosphorylation of β-arrestin2 regulates clathrin-mediated internalization of the β_2-adrenergic receptor. In contrast to β-arrestin1, which is phosphorylated by ERK1 and ERK2, phosphorylation of β-arrestin2 at Thr-383 is shown to be mediated by casein kinase II. Recently, it has been reported that phosphorylation of visual arrestin at Ser-366 prevents its binding to clathrin. Thus it appears that the function of all arrestin family members in mediating internalization of G protein-coupled receptors is regulated by distinct phosphorylation/dephosphorylation mechanisms.

The functions of β-arrestins (i.e., β-arrestin1 and β-arrestin2) in mediating desensitization and internalization of a number of G protein-coupled receptors have been studied previously in cell lines overexpressing β-arrestins (*1*) and in established mouse embryonic cell lines lacking β-arrestins (*2*). β-Arrestins bind to agonist-occupied, GRK[1]-phosphorylated G protein-coupled receptors. This inhibits second messenger signaling while initiating signaling through other pathways such as the MAP kinases (*3*). They also serve as adaptor proteins to target the receptors to clathrin-coated vesicles for internalization by binding to several molecules involved in the machinery for receptor internalization, at least including clathrin (*4*), AP-2 (5), and NSF (*6*). Overexpression of β-arrestins in a variety of cell lines augments desensitization and internalization of a number of G protein-coupled receptors (*7*). In contrast, mouse embryonic fibroblasts derived from mice lacking β-arrestins showed that agonist-induced desensitization of the β_2-adrenergic receptor and angiotensin II type 1A receptor is impaired in cells lacking either β-arrestin1 or β-arrestin2 and is impaired even more in cells lacking both β-arrestins (*2*). Interestingly, internalization of G protein-coupled receptors is differentially regulated by β-arrestin1 and β-arrestin2. Recent results from β-arrestin knockout cell lines demonstrated that internalization of the β_2-adrenergic receptor is significantly impaired in cell lines lacking β-arrestin2 but not in cell lines lacking β-arrestin1. In contrast, agonist-induced internalization of the angiotensin II type 1A receptor is slightly reduced in β-arrestin1 knockout cells but is not changed in the β-arrestin2 knockout cells. Only when both β-arrestins are disrupted is internalization of the angiotensin II type 1A receptor dramatically reduced.

Previously, we have shown that phosphorylation/dephosphorylation of β-arrestin1 regulates its function in internalization of the β_2-adrenergic receptor and its ability to promote agonist-induced ERK activation (*8*−*10*). Cytosolic β-arrestin1 is constitutively phosphorylated at Ser-412. This phosphorylation site is specific to β-arrestin1 but not other arrestin family members. Isoproterenol stimulation leads to dephosphorylation of β-arrestin1 on the plasma membrane, a process that is not required for its receptor binding and desensitization of the β_2-adrenergic receptor. However, dephosphorylation of β-arrestin1 is required for targeting the receptor to clathrin-coated vesicles for internalization (*8*) and for c-Src binding and agonist-dependent activation of ERKs (*10*). Moreover, ERKs phosphorylate β-arrestin1 at Ser-412, thereby exerting a negative feedback regulation of its function (*11*). Likewise, *Drosophila* visual arrestin is phosphorylated at the carboxy-terminal Ser-366 by an unidentified kinase (*12*). This phosphorylation site is only present in visual arrestin. Phosphorylation of visual arrestin prevents its binding to clathrin. Interestingly, in addition to arrestin family members, the function of several other molecules involved in the endocytic machinery has been shown to be regulated by

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[1] Abbreviations: AP-2, adaptor protein-2; β_2-AR, β_2-adrenergic receptor; CaMK II, calmodulin-dependent protein kinase II; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DSP, dithiobis(succinimidyl propionate); ERK, extracellular signal-regulated kinase; GRK, G protein-coupled receptor kinase; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; NSF, *N*-ethylmaleimide-sensitive factor; PAGE, polyacrylamide gel electrophoresis.

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phosphorylation/dephosphorylation events. For example, phosphorylation of the β-subunit of AP-2 prevents the association of the adaptor complexes with clathrin (*13*). In addition, phosphorylation of dynamin 1 and synaptojanin inhibits their interaction with amphiphysin, and phosphorylation of amphiphysin 1 interferes with the interaction of the amphiphysin dimer with clathrin and AP-2 (*14*). All of these data suggest that dephosphorylation and phosphorylation of these components are important for the assembly and disassembly of the clathrin-coated vesicles.

In this paper we demonstrate that phosphorylation/dephosphorylation of β-arrestin2 at the carboxy-terminal Ser-361 and Thr-383 affects its binding affinity for clathrin and regulates its function in promoting internalization of the β_2-adrenergic receptor. We have identified casein kinase II as the kinase which specifically phosphorylates Thr-383, a site that is within or very close to the β-arrestin binding domains for clathrin (*15*) and AP-2 (*16*).

MATERIALS AND METHODS

Plasmid Construction and Site-Directed Mutagenesis. An oligonucleotide, 5′-CATCACCATCACCATCAT-3′, encoding six histidine residues was engineered at the carboxy-terminal end of the rat β-arrestin2 cDNA coding sequence by polymerase chain reaction (PCR). This 1.3 kb fragment was inserted at *Kpn*I/*Xba*I sites of pcDNA3 to create pcDNA3/βarr2-6×His. The vectors expressing phosphorylation mutants of His-tagged β-arrestin2 in mammalian cells were constructed by recombination PCR as described before (*17*). The nucleotides TCA, encoding Ser at amino acid 361, and the nucleotides ACA, encoding Thr at amino acid 383, were replaced with GCA or GAC such that Ser-361 and Thr-383 were replaced with Ala or Asp. The PCR products were verified by DNA sequencing (Sequencing Facility, Duke University Cancer Center).

To express the recombinant GST fusion proteins of wild-type and S361D and T383D phosphorylation mutants of β-arrestin2-6×His, a 0.3 kb *Xho*I/*Not*I fragment of pGEX4T3/GST-βarr2, which contains the Ser-361 and Thr-383 sites, was replaced with the corresponding fragment of pcDNA3/βarr2-6×His (wild type, S361D, or T383D) with six histidine residues tagged at the carboxy terminus.

Transfection and Metabolic Labeling. The plasmids of interest were transfected into COS-7 or HEK 293 cells using FuGene 6 transfection reagent (Roche). For metabolic labeling, cells overexpressing β-arrestin2-6×His or its phosphorylation mutants were starved in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 30 min, labeled for 90 min in the same medium containing [^{32}P]orthophosphate (0.5 mCi/mL), and harvested for β-arrestin2-6×His purification.

Purification of β-Arrestin2-6×His. To purify cellular β-arrestin2-6×His, COS-7 cells overexpressing β-arrestin2-6×His were harvested and lysed in binding buffer (20 mM Tris, pH 7.9, 0.5 M NaCl, 5 mM imidazole) containing a mixture of protease inhibitors and 0.2% Nonidet P-40. To purify phospho-β-arrestin2-6×His, 50 mM NaF and 10 mM sodium pyrophosphate were also added to inhibit phosphatase activity. β-Arrestin2-6×His was purified by nickel affinity chromatography as described before (*8*).

GST fusion proteins of β-arrestin2 were purified as follows. BL21(DE3)(LysS) cells transformed with pGEX4T3/GST-βarr2-6×His, pGEX4T3/GST-(S361D)βarr2-6×His, and pGEX4T3/GST-(T383D)βarr2-6×His were treated with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 2 h and harvested. Bacterial pellets were lysed and subjected to nickel affinity chromatography as described above for the purification of cellular β-arrestin2-6×His. After elution, the buffer was dialyzed against phosphate-buffered saline (PBS) containing protease inhibitor cocktails. Glutathione−Sepharose beads were added and gently agitated at 4 °C for 2 h. Beads were washed three times in ice-cold PBS. The integrity of the fusion proteins was analyzed by SDS−polyarylamide gel electrophoresis (SDS−PAGE) and Coomassie blue staining.

Phosphoamino Acid Analysis and Phosphopeptide Sequencing. Purified phosphorylated β-arrestin2-6×His or GST fusion proteins of β-arrestin2-6×His were fractionated by SDS−PAGE, transferred to poly(vinylidene difluoride) membranes (Immobilon PVDF, Millipore), and then eluted as described (*18*). Proteins were hydrolyzed in 6 N HCl for 1 h at 110 °C, lyophilized, combined with the standards of phosphoserine, phosphothreonine, and phosphotyrosine, and fractionated by one-dimensional thin-layer elctrophoresis as described (*18*, *19*). Phosphoamino acid standards were stained with ninhydrin, and the ^{32}P-labeled phosphoamino acids were detected by autoradiography.

To identify the phosphorylation sites of β-arrestin2 in COS-7 cells, ^{32}P-labeled β-arrestin2-6×His was purified from six confluent 15 cm plates of COS-7 cells overexpressing β-arrestin2-6×His, subjected to SDS−PAGE, and transferred to Immobilon PVDF membrane (Millipore). The phosphorylated bands were cut out, digested in situ with sequencing grade trypsin, and purified by reverse-phase HPLC. The amino acid sequencing was performed using an Applied Biosystems model 477A protein sequencer with an in-line 120A PTH analyzer (Protein Chemistry Core Facility, Baylor College of Medicine).

In Vitro Phosphorylation of GST Fusion Proteins of β-Arrestin2-6×His by Different Kinases. One microgram of purified GST protein or GST fusion proteins of wild-type, S361D, and T383D β-arrestin2-6×His was incubated with 0.5 μL (activity: 500000 units/mL) of recombinant human casein kinase II (Calbiochem), calmodulin-dependent protein kinase II (CaMKII) (New England Biolabs), protein kinase C (New England Biolabs), or 20 μg/mL purified G protein-coupled receptor kinase 5 (GRK5) along with 10 μCi of [γ-^{32}P]ATP (NEN) and 100 μM ATP in the reaction buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol and additional 2 mM $CaCl_2$, 1.2 mM calmodulin for CaMKII) at 30 °C for 30 min. The phosphorylated proteins were fractionated by SDS−PAGE, transferred to the Immobilon PVDF membrane, and exposed for autoradiography.

Receptor Binding and Desensitization Assays. COS-7 cells were transiently transfected with pcDNA1/FLAG-β_2AR and either an empty CMV vector (mock) or one of the β-arrestin2-6×His expression vectors (wild type or β-arrestin2 phosphorylation mutants). Two days after transfection, cells were serum-starved for 30 min, incubated with or without 10 μM (−)-isoproterenol for 2 min, and then treated with dithiobis(succinimidyl propionate) (Pierce) for cross-linking. Cells were harvested for coimmunoprecipitation of FLAG-tagged β_2-adrenergic receptor and β-arrestin2-6×His (wild type or phosphorylation mutants) as described for the receptor

binding of β-arrestin1 before (*8*). The immunoprecipitate of FLAG-β_2-AR was fractionated by SDS−PAGE and transferred to the nitrocellulose membrane. The immunoblot was detected with anti-His antibody (Santa Cruz Inc.).

For desensitization assays, cells described above were incubated with 1 μCi/mL [^{3}H]adenine overnight and then treated with or without 10 μM (−)isoproterenol at different periods of time (0, 1, 2, 5, 10, 20, and 30 min). The agonist-stimulated conversion of [^{3}H]adenine to [^{3}H]cAMP in whole cells was determined as described (*20*).

Agonist-Promoted Internalization of the β_2-Adrenergic Receptor. COS-7 cells were transiently transfected with pcDNA1/FLAG-β_2AR and either an empty vector or one of the β-arrestin2-6×His expression vectors (wild type or phosphorylation mutants). Two days later, cells were starved in serum-free medium for 30 min and then incubated with 10 μM (−)-isoproterenol for 30 min. Internalization of the β_2-adrenergic receptor was determined by flow cytometry as described previously (*21*).

Coimmunoprecipitations. An empty CMV vector or one of the expression vectors encoding wild type or phosphorylation mutants of β-arrestin2-6×His was transfected into HEK 293 cells. Two days later cells were harvested and dissolved in the lysis buffer for coimmunoprecipitation as described previously (*8*). β-Arrestin2-6×His was immunoprecipitated using anti-His antibody-conjugated agarose (Santa Cruz Inc.), resolved by SDS−PAGE, and transferred to nitrocellulose membranes for immunoblotting. The endogenous clathrin heavy chain and β-adaptin were detected using monoclonal antibodies specific to clathrin and β-adaptin (Transduction Laboratories), respectively.

Coimmunoprecipitation of FLAG-tagged β-arrestin2 with endogenous casein kinase II was carried out in COS-7 cells transfected with an empty vector or the expression vector encoding FLAG-tagged β-arrestin2. Cells were treated with dithiobis(succinimidyl propionate) (Pierce) for cross-linking. β-Arrestin2 was immunoprecipitated with anti-FLAG antibody-conjugated agarose (Sigma), resolved by SDS−PAGE, and transferred to nitrocellulose membranes for Western blot analysis using a monoclonal antibody specific to casein kinase II β-subunits (Transduction Laboratories).

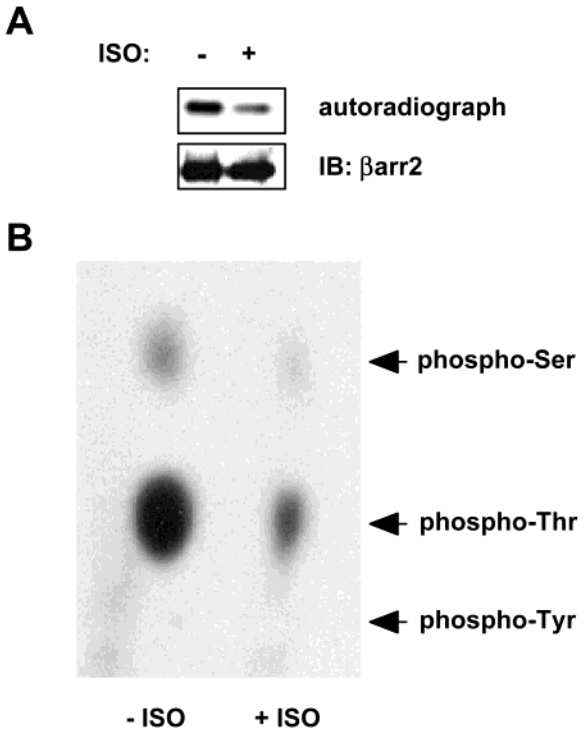

FIGURE 1: Agonist-stimulated dephosphorylation of β-arrestin2 at serine and threonine residues. (A) Phosphorylation of β-arrestin2 is reduced by isoproterenol stimulation. COS-7 cells transiently transfected with the expression plasmids for the β_2-adrenergic receptor and β-arrestin2-6×His were split to two plates and then labeled with [^{32}P]orthophosphate in serum-free, phosphate-free medium for 90 min. Cells were treated with or without 10 μM (−)-isoproterenol (ISO) for 5 min and harvested. His-tagged β-arrestin2 was purified by nickel affinity beads. The proteins were fractionated by SDS−PAGE, transferred to Immobilon PVDF membrane, and exposed for autoradiography. The top panel is the 50 kDa band of ^{32}P-labeled β-arrestin2-6×His. After the autoradiograph was developed, the membrane was subjected to immunoblotting. The bottom panel is the immunoblot (IB) showing total β-arrestin2-6×His (βarr2) detected by the anti-βarr2 antibody. (B) Phosphoamino acid analysis of phosphorylated β-arrestin2-6×His in the presence or absence of isoproterenol treatment. The ^{32}P-labeled β-arrestin2-6×His bands were cut out for one-dimensional phosphoamino acid analysis as described in Materials and Methods. The positions of phosphorylated serine, threonine, and tyrosine standards are marked with arrows.

RESULTS

Agonist Stimulation Promotes Dephosphorylation of β-Arrestin2. Previously, we have shown that cytosolic β-arrestin1 is constitutively phosphorylated. Agonist stimulation leads to the translocation of β-arrestin1 to the plasma membrane where it is dephosphorylated (*8*). To determine whether cellular β-arrestin2 is also a phosphoprotein, we first overexpressed β-arrestin2 in either HEK 293 cells or COS-7 cells and assessed its phosphorylation status by metabolic labeling with [^{32}P]orthophosphate. The results show that cellular β-arrestin2 is a highly phosphorylated protein (Figure 1). To investigate whether β-arrestin2 phosphorylation is regulated by agonist stimulation, we transfected expression vectors for the FLAG-tagged β_2-adrenergic receptor and His-tagged β-arrestin2 into COS-7 cells and assessed the phosphorylation status of β-arrestin2 with or without agonist stimulation. Cells were metabolically labeled with [^{32}P]-orthophosphate for 90 min. After isoproterenol treatment for 5 min His-tagged β-arrestin2 from whole cell extracts was purified by nickel affinity chromatography and subjected to SDS−PAGE analysis. As shown in Figure 1A, isoproterenol stimulation for 5 min leads to a 50% reduction of β-arrestin2 phosphorylation in the whole cell extracts of COS-7 cells. Similar results were observed with immunoprecipitated FLAG-tagged β-arrestin2 from both COS-7 and HEK 293 cells (data not shown). Previously, we have shown that, under these conditions, there is only a 20% reduction of β-arrestin1 phosphorylation in the whole cell extracts of HEK 293 cells (*8*). This result suggests that β-arrestin2 may be more efficiently dephosphorylated than β-arrestin1 after agonist stimulation.

Next the phosphorylated β-arrestin2 bands shown in Figure 1A were cut out for phosphoamino acid analysis. β-Arrestin2 is mostly phosphorylated at threonine residues and less phosphorylated at serine residues (Figure 1B). In a series of experiments the ratio of phosphorylated threonine to serine was about 2−3:1. Isoproterenol treatment for 5 min significantly reduced phosphorylation of both serine and threonine

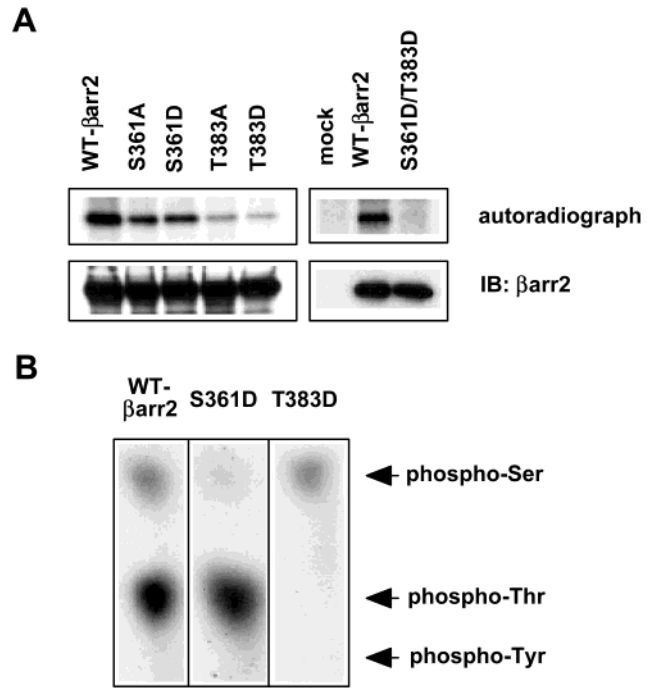

FIGURE 2: Substitution of Ser-361 and/or Thr-383 of β-arrestin2 with Ala (A) or Asp (D) inhibits phosphorylation of β-arresitn2. (A) An empty vector (mock) or an expression vector encoding wild-type (WT), single mutant (S361A, S361D, T383A, T383D), or double mutant (S361D/T383D) β-arrestin2-6×His was transiently transfected into COS-7 cells. Cells were labeled with [^{32}P]-orthophosphate for 90 min and harvested for SDS−PAGE analysis, autoradiography, and immunoblotting. The top panel is the autoradiograph of phosphorylated β-arrestin2-6×His and the bottom panel is total β-arrestin2-6×His from the same blots detected by its specific antibody. (B) Phosphoamino acid analysis of phosphorylated wild-type β-arrestin2-6×His and its phosphorylation mutants, S361D and T383D. The ^{32}P-labeled β-arrestin2-6×His (WT, S361D, and T383D) bands were cut out for phosphoamino acid analysis as described above.

residues. Thus, β-arrestin2 phosphorylation differs from that of β-arrestin1, which is phosphorylated almost exclusively at serine residues.

β-Arrestin2 Is Phosphorylated at the Carboxy-Terminal Ser-361 and Thr-383. The trypsin-digested phospho-β-arrestin2 purified from COS-7 cells overexpressing His-tagged β-arrestin2 was analyzed by reverse-phase HPLC, followed by protein sequencing of the major phosphopeptide. The result revealed that the carboxy-terminal Ser-361 and Thr-383 are the phosphorylation sites of β-arrestin2. To confirm that these two sites are the relevant physiological phosphorylation sites of β-arrestin2, we generated a number of expression vectors of His-tagged β-arrestin2 mutants in which Ser-361 and/or Thr-383 were (was) substituted with Ala or Asp. These mutants were transfected into COS-7 cells individually, and their phosphorylation status was examined. As shown in Figure 2A, the expression levels of wild-type or mutant β-arrestin2 are comparable. However, β-arrestin2 phosphorylation was partially reduced when Ser-361 was mutated to Ala (S361A) or Asp (S361D) and was dramatically reduced when Thr-383 was replaced with Ala (T383A) or Asp (T383D). β-Arrestin2 phosphorylation was almost completely abolished when both Ser-361 and Thr-383 were mutated to Asp (S361D/T383D). Further examination by phosphoamino acid analysis of S361D and T383D β-arrestin2-6×His mutants confirmed that β-arrestin2 is predominantly phosphorylated at Thr-383 and less phosphorylated at Ser-361 (Figure 2B).

To identify candidate kinases that might phosphorylate β-arrestin2 at Ser-361 and Thr-383, we tried to phosphorylate GST fusion proteins of wild-type, S361D, or T383D β-arrestin2-6×His in vitro by different potential candidate kinases, including casein kinase I, casein kinase II, calmodulin-dependent protein kinase II, protein kinase C, GRK2, and GRK5. The β-arrestin2 phosphorylation site, Thr-383, is located within an acidic domain (TDDD), a consensus phosphorylation site for casein kinase II, GRK2, or GRK5. We found that casein kinase II was capable of phosphorylating GST−wild-type β-arrestin2-6×His and GST−S361D β-arrestin2-6×His but not GST−T383D β-arrestin2-6×His (Figure 3A). Phosphoamino acid analysis further confirmed that casein kinase II phosphorylated GST−β-arrestin2-6×His and GST−S361D β-arrestin2-6×His solely at threonine residues, which was completely abolished when Thr-383 of GST−β-arrestin2-6×His was replaced with Asp (Figure 3B). These results suggest that casein kinase II is a potential candidate for phosphorylating β-arrestin2 at Thr-383 in vitro. Other kinases such as calmodulin-dependent protein kinase II, GRK5, protein kinase C, and casein kinase I were able to phosphorylate wild-type GST−β-arrestin2 as well as S361D and T383D of GST−β-arrestin2 (Figure 3A), indicating that these kinases are not candidate kinases for phosphorylating β-arrestin2 at Ser-361 or Thr-383. The purified GRK2 does not phoshorylate β-arrestin2 in vitro.

To assess whether casein kinase II is the potential kinase which phosphorylates β-arrestin2 in cells, we tried to block β-arrestin2 phosphorylation in COS-7 cells using the chemical inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which has been shown to block the activity of casein kinase II and RNA polymerase II. Unfortunately, however, DRB treatment for 2 h caused a proportional decrease of phosphorylation and expression levels of β-arrestin2 such that we could not assess its ability to inhibit phosphorylation of β-arrestin2 in COS-7 cells (data not shown). However, we were able to detect coimmunoprecipitation of the endogenous casein kinase II with FLAG-tagged β-arrestin2 in COS-7 cells (Figure 3C) and HEK 293 cells in an agonist-independent manner (data not shown). In contrast, casein kinase II is not the kinase responsible for β-arrestin1 phosphorylation in cells. In similar experiments casein kinase II did not coimmunoprecipitate with β-arrestin1 in COS-7 cells or HEK 293 cells either (data not shown). The kinase which phosphorylates β-arrestin2 at Ser-361 has not yet been identified.

Point Mutations of β-Arrestin2 at Ser-361 or Thr-383 Do Not Affect Its Receptor Binding and Its Ability To Promote Desensitization of the β_2-Adrenergic Receptor. To assess the effect of β-arrestin2 phosphorylation on its function in receptor signaling, we first examined the ability of wild-type or mutants of β-arrestin2 to bind to the β_2-adrenergic receptor in the presence or absence of isoproterenol treatment. The Ala and Asp mutants of β-arrestin2 are predicted to mimic the dephosphorylated and phosphorylated forms, respectively. Similar to β-arrestin1, isoproterenol stimulation for 2 min promoted the association of β-arrestin2 and the β_2-adrenergic receptor in COS-7 cells. Receptor binding was not affected by mutation of Ser-361 or Thr-383 to Ala or

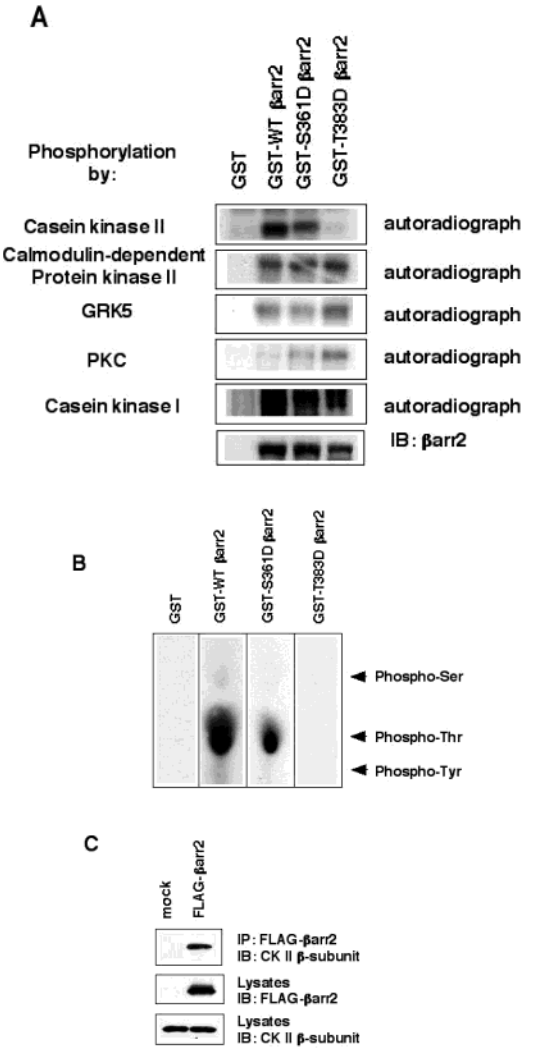

FIGURE 3: Casein kinase II binds to cellular β-arrestin2 and mediates Thr-383 phosphorylation of GST-β-arrestin2-6×His. (A) Phosphorylation of GST−β-arrestin2-6×His in vitro. One microgram of purified recombinant GST or GST fusion proteins of β-arrestin2-6×His (WT, S361D, or T383D) was subjected to in vitro phosphorylation by casein kinase II, calmodulin-dependent protein kinase II, GRK5, protein kinase C, or casein kinase I. The GST protein or GST fusion proteins of β-arrestin2-6×His were pulled down, resolved by SDS−PAGE, and transferred to Immobilon PVDF membrane. The top five panels are the autoradiograph of phosphorylated β-arrestin2-6×His, and the bottom panel is the immunoblot of β-arrestin2-6×His probed with anti-βarr2 antibody. (B) Phosphoamino acid analysis of the GST fusion protein of β-arrestin2-6×His phosphorylated by casein kinase II. The casein kinase II-phosphorylated β-arrestin2-6×His (WT, S361D, T383D) was subjected to phosphoamino acid analysis as described in Figure 2. (C) Cellular β-arrestin2 interacts with endogenous casein kinase II. COS-7 cells were transiently transfected with an empty vector (mock) or the expression vector for FLAG-tagged β-arrestin2. β-Arrestin2 was immunoprecipitated by the anti-FLAG M2 monoclonal antibody from the whole cell extracts treated with dithiobis(succinimidyl propionate). Proteins were fractionated by SDS−PAGE and transferred to nitrocellulose membranes. The immunoblot was probed with a monoclonal antibody specific to the casein kinase II (CK II) β-subunit as shown on the top panel. The middle and bottom panels are immunoblots of FLAG-tagged β-arrestin2 and endogenous casein kinase II from the whole cell lysates.

Asp (Figure 4A). Even when both phosphorylation sites were mutated to Asp, the β-arrestin2 mutant was still able to bind to the receptor (Figure 4A). Similar results were observed in HEK 293 cells transfected with wild type or mutants of

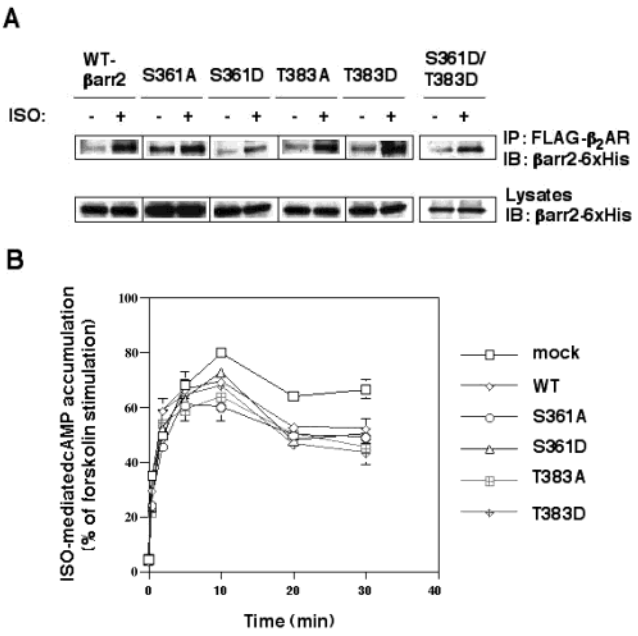

FIGURE 4: Receptor binding of β-arrestin2 and desensitization of the β_2-adrenergic receptor are not affected by mutation of phosphorylation sites of β-arrestin2. (A) Isoproterenol-promoted association of β-arrestin2 and the β_2-adrenergic receptor. COS-7 cells were transiently transfected with the FLAG-tagged β_2-adrenergic receptor (β_2-AR) and one of the β-arrestin2-6×His expression vectors (WT, S361A, S361D, T383A, T383D, or S361D/T383D). Cells were starved in serum-free medium for 30 min and then treated with 10 μM (−)-isoproterenol for 2 min followed by the addition of dithiobis(succinimidyl propionate) (DSP) for cross-linking. β_2-AR was immunoprecipitated with the anti-FLAG M2 monoclonal antibody. After SDS−PAGE, coimmunoprecipitated β-arrestin2-6×His was detected using a specific anti-His antibody (top panel). The bottom panel is an immunoblot of total β-arrestin2-6×His from the whole cell lysates. (B) Phosphorylation of β-arrestin2 does not affect its ability to promote agonist-induced desensitization of the β_2-adrenergic receptor. COS-7 cells transfected with the FLAG-β_2-AR and one of the β-arrestin2-6×His expression vectors were treated with (−)-isoproterenol (10 μM) for different periods of time (0, 1, 2, 5, 10, 20, and 30 min). Isoproterenol-induced cAMP accumulation in the whole cells was determined as the percent conversion of [^{3}H]adenine into [^{3}H]cAMP and then normalized to total forskolin (50 μM) stimulated cAMP accumulation for each cell line. Data shown are representative of three independent experiments. The error bars represent the standard deviation of triplicates in this experiment.

β-arrestin2 (data not shown). Likewise, the ability of β-arrestin2 to promote isoproterenol-induced desensitization of the β_2-adrenergic receptor was not significantly changed by mutating Ser-361 or Thr-383 to Ala or Asp (Figure 4B).

Phosphorylation of β-Arrestin2 Regulates Its Affinity for Binding to Clathrin and Affects Internalization of the β_2-Adrenergic Receptor. It has been demonstrated that β-arrestins target the β_2-adrenergic receptor to clathrin-coated vesicles for internalization (*4*). To investigate if β-arrestin2 phosphorylation regulates its function in agonist-mediated internalization of the β_2-adrenergic receptor, we assessed the association of β-arrestin2 phosphorylation mutants with clathrin. In *in vitro* experiments, Sepharose beads conjugated with GST fusion proteins of wild-type, S361D, and T383D β-arrestin2-6×His or control GST protein were incubated with equal amounts of purified clathrin at 4 °C for 2 h. The GST protein complex was precipitated and fractionated by SDS−PAGE analysis. In the GST pull-down experiments,

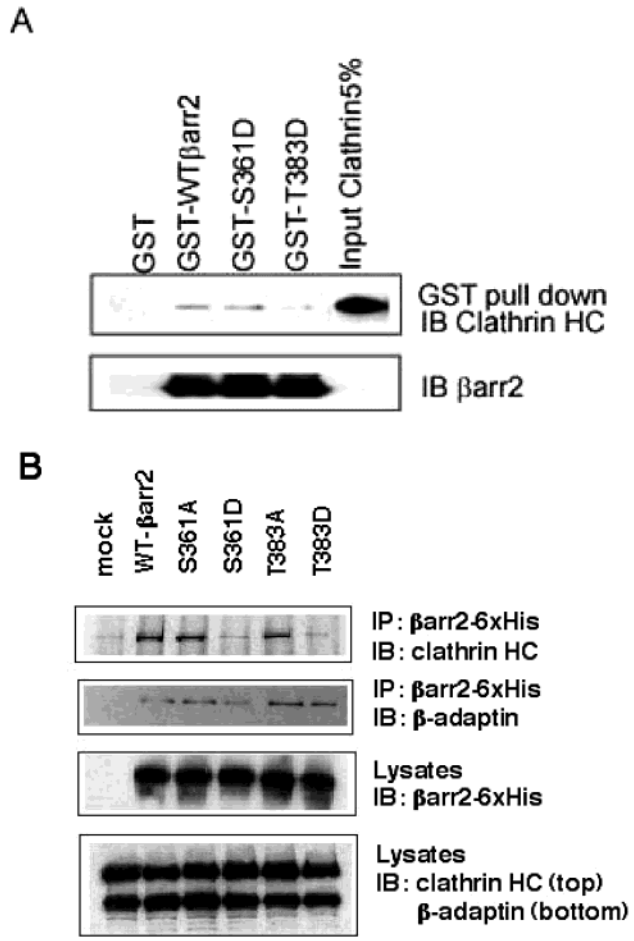

FIGURE 5: Association of β-arrestin2 with clathrin, but not β-adaptin, is regulated by β-arrestin2 phosphorylation. (A) Interaction of GST fusion proteins of β-arrestin2-6×His with purified clathrin. Purified clathrin (2 μg) was incubated with 3 μg of GST protein or the GST fusion protein of β-arrestin2-6×His (WT, S361D, or T383D) at 4 °C for 2 h. The GST beads were pulled down and washed four times in PBS. Proteins on the beads were resolved by SDS−PAGE and transferred to nitrocellulose membrane. The immunoblot was probed with an antibody specific to the clathrin heavy chain (top panel). The bottom panel shows the expression levels of GST−β-arrestin2-6×His, GST−S361D β-arrestin2-6×His, and GST−T383D β-arrestin2-6×His pulled down by GST beads. (B) Coimmunoprecipitation of cellular β-arrestin2 with endogenous clathrin and β-adaptin. HEK 293 cells were transiently transfected with expression vectors of His-tagged β-arrestin2, WT or mutants. β-Arrestin2 was immunoprecipitated with the anti-His antibody-conjugated agarose. The endogenous clathrin heavy chain (HC) and β-adaptin were detected by clathrin and β-adaptin antibodies as shown in the top two panels. The bottom two panels are immunoblots of β-arrestin2-6×His and endogenous clathrin and β-adaptin from the whole cell lysates.

the mutant T383D did not bind clathrin as well as the wild-type β-arrestin2 (Figure 5A, top panel). In three independent experiments about 50% deficiency in pulling down clathrin was seen, when compared with β-arrestin2. These results suggest that phosphorylation of β-arrestin2 at Thr-383 decreases its binding affinity for clathrin *in vitro*. The S361 mutant did not appear impaired in its ability to bind clathrin in these experiments (but see Figure 5B below).

To examine the effect of β-arrestin2 phosphorylation on its binding to clathrin and β-adaptin in cells, we transfected His-tagged β-arrestin2, wild type or phosphorylation mutants, into HEK 293 cells. Our results showed that endogenous clathrin was present in the immunoprecipitates of wild-type β-arrestin2 and Ala mutants (S361A, T383A) of β-arrestin2 (Figure 5B, top panel). In contrast, we detected much less association of endogenous clathrin with the Asp mutants (S361D and T383D) of β-arrestin2, which mimic the phosphorylated form of β-arrestin2. In four experiments, the mutant forms of β-arrestin2 bound only 47 ± 16% as much clathrin as the wild-type β-arrestin2. The fact that S361D was impaired in clathrin binding in the cellular experiment (Figure 5B) suggests that S361 may be of regulatory importance in clathrin interactions. It is presently unclear why this result was not obtained in the *in vitro* pull-down experiment (Figure 5A). The coimmunoprecipitation of β-adaptin and β-arrestin2 was not significantly altered by mutation of Ser-361 or Thr-383 to Ala or Asp (Figure 5B, second panel). These results suggest that point mutation of Ser-361 and Thr-383 to Asp might regulate the affinity of β-arrestin for clathrin, but not β-adaptin, in HEK 293 cells.

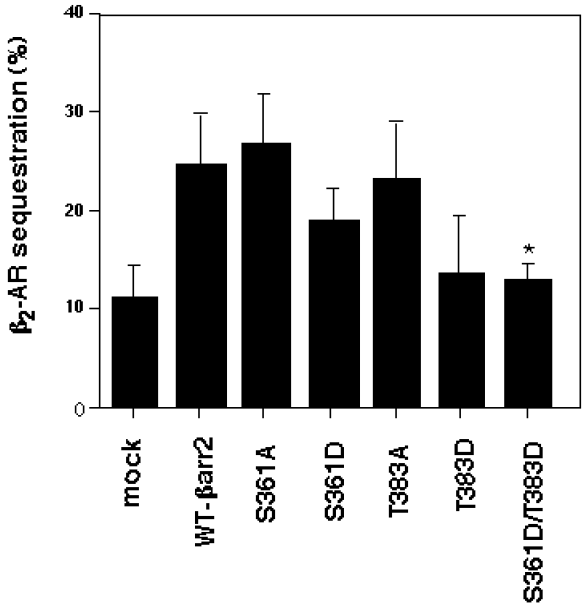

FIGURE 6: Effect of β-arrestin2 phosphorylation on internalization of the β_2-adrenergic receptor. The expression vector of FLAG-β_2-AR was cotransfected with that of β-arrestin2-6×His (WT or mutants) into COS-7 cells. Cells were starved for 30 min, followed by (−)-isoproterenol (10 μM) treatment for 30 min. Receptor internalization was assessed by flow cytometry as described previously. Data shown are the mean ± SEM of four independent experiments done in duplicate. *: p value < 0.02 as compared with WT β-arrestin2 (Student's t test).

To understand if phosphorylation of β-arrestin2 regulates its function in receptor internalization, we transfected His-tagged β-arrestin2, wild type or mutants, with the FLAG-tagged β_2-adrenergic receptor into COS-7 cells and measured isoproterenol-induced internalization. Wild-type β-arrestin2 significantly promoted isoproterenol-induced internalization of the β_2-adrenergic receptor in COS-7 cells (Figure 6). This ability was not affected by point mutation of β-arrestin2 phosphorylation sites to Ala (S361A and T383A). In contrast, compared to the wild-type β-arrestin2, the Asp mutants of β-arrestin2, especially the T383D mutant and the S361D/T383D double mutant, showed reduced ability to promote agonist-mediated receptor internalization in COS-7 cells. These results are consistent with the finding that the Asp mutants of β-arrestin2 bind less well to clathrin (Figure 5).

In these internalization experiments, all of the Asp mutants of β-arrestin2 did not show a significant dominant-negative effect on agonist-induced internalization of the β_2-adrenergic receptor in COS-7 cells (Figure 6). This might be explained by the result (Figure 5B) that the association of β-adaptin with β-arrestin2 was not significantly altered by mutation of β-arrestin2 at Ser-361 or Thr-383.

DISCUSSION

Previously, it has been shown that the carboxy terminus of β-arrestin2, comprising amino acids 378−410, is involved in binding to the β_2 subunit of AP-2 and is required for targeting the β_2-adrenergic receptor to clathrin-coated pits (*16*). The binding for clathrin is localized within amino acids 371−379 (*15*). In this paper we have demonstrated that isoproterenol stimulation leads to dephosphorylation of β-arrestin2 at the carboxy-terminal Ser-361 and Thr-383, which are within or adjacent to the binding domain for clathrin. Phosphorylation/dephosphorylation of β-arrestin2 affects its affinity for binding to clathrin and regulates its function in internalization of the β_2-adrenergic receptor. The association of β-arrestin2 with clathrin is significantly reduced when Thr-383 or Ser-361 is substituted with Asp but not Ala. Consistent with this finding, the function of T383D and S361D, but not T383A or S361A, β-arrestin2 is impaired in promoting internalization of the β_2-adrenergic receptor. However, we did not observe a dominant-negative role for the Asp mutant of β-arrestin2 in receptor internalization. This result suggests that these mutants retain the affinity for binding to β-adaptin, which is sufficient to target the receptor to clathrin-coated pits for internalization. This is consistent with a previous result showing that the binding of a receptor−β-arrestin2 complex to AP-2, but not to clathrin, is necessary for the initial targeting of the β_2-adrenergic receptor to clathrin-coated pits (*16*). Different from β-arrestin2, previously we have shown that the S412D mutant of β-arrestin1 does not bind to clathrin and acts as a dominant-negative inhibitor in clathrin-mediated internalization of the β_2-adrenergic receptor (*8*). Similarly, in *Drosophila*, dephosphorylation of visual arrestin at Ser-366 is required for clathrin binding (*12*). All of these results suggest that dephosphorylation of arrestin family members at carboxy-terminal Ser or Thr site(s) regulates their function in promoting internalization of G protein-coupled receptors.

Compared to β-arrestin1, β-arrestin2 has a 6-fold higher affinity for clathrin (*4*). Thus, β-arrestin2 is more potent than β-arrestin1 in promoting internalization of the β_2-adrenergic receptor. In addition, β-arrestin2 is more efficient at translocating to the plasma membrane to bind to the receptor (*22*). These findings correlated with our observation that agonist stimulation leads to greater dephosphorylation of β-arrestin2 (50%) than β-arrestin1 (20%) in whole cell extracts. Thus far, the mechanisms triggering the agonist-dependent dephosphorylation of β-arrestins and translocation of β-arrestins from cytosol to plasma membrane are not yet clear. Previously, it has been shown that the high-affinity stoichiometric binding of purified β-arrestin to the β_2-adrenergic receptor occurs in a GRK-dependent manner (*23*). Thus, the agonist-induced translocation of β-arrestins from cytosol to plasma membrane and binding of β-arrestins to the receptor are at least partly mediated by GRK phosphorylation of the β_2-adrenergic receptor. It is not yet clear whether dephosphorylation of β-arrestins occurs before or after binding to the receptor. However, it seems plausible that binding of β-arrestins to the phosphorylated receptor positions it in proximity to the relevant phosphatase or alters its conformation such that it becomes a substrate for the phosphatase. The identification of the phosphatase(s) that specifically dephosphorylate(s) β-arrestins in response to agonist stimulation will be of great importance to understand the functional regulation of β-arrestins in GPCR signaling.

Casein kinase II-like activity has been shown to be associated with clathrin-coated vesicles (*24*, *25*) and is required for continuous endocytosis of the transferrin receptor (*26*). It has been shown that the casein kinase II-like activity present in clathrin-coated vesicles can phosphorylate clathrin light chains (*24*). In addition, the clathrin-coated vesicle-associated kinase(s) can phosphorylate a fusion protein containing an optimized casein kinase II recognition site (-DSDDDDD-) (*25*), suggesting the presence of a casein kinase II-like activity in the vesicles. We postulate that this casein kinase II-like activity associated with clathrin-coated vesicles is capable of phosphorylating β-arrestin2, a process that is required for dissociation of β-arrestin2 from clathrin-coated vesicles once the receptor is internalized. Similar to ERK-mediated phosphorylation of β-arrestin1, phosphorylation of β-arrestin2 by casein kinase II or a casein kinase II-like activity may exert a negative feedback regulation of its function. All of these results thus indicate that clathrin-mediated internalization of G protein-coupled receptors is regulated by phosphorylation/dephosphorylation of arrestin family members, which is controlled by different phosphorylation modifications.

Quite recently, Kim et al. (*27*) have also reported phosphorylation of T^{382} in bovine β-arrestin2 by casein kinase II, in agreement with our findings. In contrast, however, they did not find evidence for regulation of arrestin function in internalization or in binding clathrin. While there are small differences in the experimental systems used (COS-1 vs COS-7 cells, T → E vs T → D mutation, bovine vs rat β-arrestin), these seem unlikely to explain the differences. It should be noted, however, that the most marked impairment in internalization we noted was with the double mutant ($S^{361}T^{383}$ → D). Phosphorylation of S^{361} was not explored in the study by Kim et al., and it is possible that it, as well as T^{383}, is involved in regulating β-arrestin2 function.

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REFERENCES

1. Lefkowitz, R. J. (1998) *J. Biol. Chem. 273*, 18677−18680.
2. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 1601−1606.
3. Pierce, K. L., Luttrell, L. M., and Lefkowitz, R. J. (2001) *Oncogene 20*, 1532−1539.
4. 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.

5. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3712−3717.
6. McDonald, P. H., Cote, N. L., Lin, F. T., Premont, R. T., Pitcher, J. A., and Lefkowitz, R. J. (1999) *J. Biol. Chem. 274*, 10677−10680.
7. Ferguson, S. S. (2001) *Pharmacol. Rev. 53*, 1−24.
8. Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem. 272*, 31051−31057.
9. Lin, F. T., Daaka, Y., and Lefkowitz, R. J. (1998) *J. Biol. Chem. 273*, 31640−31643.
10. 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.
11. Lin, F. T., Miller, W. E., Luttrell, L. M., and Lefkowitz, R. J. (1999) *J. Biol. Chem. 274*, 15971−15974.
12. Kiselev, A., Socolich, M., Vinos, J., Hardy, R. W., Zuker, C. S., and Ranganathan, R. (2000) *Neuron 28*, 139−152.
13. Wilde, A., and Brodsky, F. M. (1996) *J. Cell Biol. 135*, 635−645.
14. Slepnev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D., and Camilli, P. D. (1998) *Science 281*, 821−824.
15. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem. 272*, 15017−15022.
16. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J. Biol. Chem. 275*, 23120−23126.
17. Jones, D. H., and Winistorfer, S. C. (1992) *BioTechniques 12*, 528−530, 532, 534−525.
18. Kamps, M. P., and Sefton, B. M. (1989) *Anal. Biochem. 176*, 22−27.
19. Hirano, A. A., Greengard, P., and Huganir, R. L. (1988) *J. Neurochem. 50*, 1447−1455.
20. Freedman, N. J., Liggett, S. B., Drachman, D. E., Pei, G., Caron, M. G., and Lefkowitz, R. J. (1995) *J. Biol. Chem. 270*, 17953−17961.
21. Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. (1994) *J. Biol. Chem. 269*, 2790−2795.
22. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) *J. Biol. Chem. 275*, 17201−17210.
23. Sohlemann, P., Hekman, M., Puzicha, M., Buchen, C., and Lohse, M. J. (1995) *Eur. J. Biochem. 232*, 464−472.
24. Hill, B. L., Drickamer, K., Brodsky, F. M., and Parham, P. (1988) *J. Biol. Chem. 263*, 5499−5501.
25. Crump, C. M., and Banting, G. (1999) *FEBS Lett. 444*, 195−200.
26. Cotlin, L. F., Siddiqui, M. A., Simpson, F., and Collawn, J. F. (1999) *J. Biol. Chem. 274*, 30550−30556.
27. Kim, Y. M., Barak, L. S., Caron, M. G., and Benovic, J. L. (2002) *J. Biol. Chem. 277*, 16837−16846.

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