Regulation of Arrestin-3 Phosphorylation by Casein Kinase II*

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Arrestins play an important role in regulating the function of G protein-coupled receptors including receptor desensitization, internalization, down-regulation, and signaling via nonreceptor tyrosine kinases and mitogen-activated protein kinases. Previous studies have revealed that arrestins themselves are also subject to regulation. In the present study, we focused on identifying potential mechanisms involved in regulating the function of arrestin-3. Using metabolic labeling, phosphoamino acid analysis, and mutagenesis studies, we found that arrestin-3 is constitutively phosphorylated at Thr-382 and becomes dephosphorylated upon β2-adrenergic receptor activation in COS-1 cells. Casein kinase II (CKII) appears to be the major kinase mediating arrestin-3 phosphorylation, since 1) Thr-382 is contained within a canonical consensus sequence for CKII phosphorylation and 2) wild type arrestin-3 but not a T382A mutant is phosphorylated by CKII in vitro. Functional analysis reveals that mutants mimicking the phosphorylated (T382E) and dephosphorylated (T382A or T382V) states of arrestin-3 promote β2-adrenergic receptor internalization and bind clathrin, β-adaptin, and Src to comparable levels as wild type arrestin-3. This suggests that the phosphorylation of arrestin-3 does not directly regulate interaction with endocytic (clathrin, β-adaptin) or signaling (Src) components and is in contrast to arrestin-2, where phosphorylation appears to regulate interaction with clathrin and Src. However, additional analysis reveals that arrestin-3 phosphorylation may regulate formation of a large arrestin-3-containing protein complex. Differences between the regulatory roles of arrestin-2 and -3 phosphorylation may contribute to the different cellular functions of these proteins in G protein-coupled receptor signaling and regulation.

Arrestins are cytosolic proteins primarily involved in the regulation of GPCR function (1, 2). Arrestins were initially described to attenuate or arrest intracellular signaling of GPCRs such as rhodopsin and β2AR. In the continuous presence of a stimulus, many cellular receptors lose their responsiveness to stimuli through a process known as desensitization. Desensitization of GPCRs is largely mediated by two protein families, G protein-coupled receptor kinases (GRKs) and arrestins. GRKs specifically phosphorylate agonist-activated receptors. Subsequently, arrestins translocate from the cytosol and bind to plasma membrane-localized phosphorylated receptors. Binding of arrestin to the intracellular surface of receptors physically blocks interaction of the receptor with its cognate heterotrimeric G protein, thus terminating signal transduction.

To date, four mammalian members of the arrestin family have been identified. The prototypical arrestin, arrestin-1 (visual arrestin), regulates light-dependent signal transduction of rhodopsin in rod photoreceptor cells (3). Another retina-specific arrestin, arrestin-4 (cone arrestin or X-arrestin), is enriched in cone photoreceptor cells but has not been further characterized (4). The nonvisual arrestins, arrestin-2 (β-arrestin-1) and arrestin-3 (β-arrestin-2), are expressed ubiquitously and are involved in desensitization of numerous GPCRs (1, 5, 6).

Recent studies reveal that the role of arrestins in GPCR regulation is not limited to receptor desensitization. Nonvisual arrestins have also been demonstrated to mediate internalization (endocytosis) of various GPCRs. Both arrestin-2 and-3 directly bind clathrin and AP2, the two major structural components of the clathrin-based endocytic machinery (7, 8). By doing so, they target receptors to clathrin-coated pits and promote receptor internalization. Nonvisual arrestins additionally mediate interaction with the GDP-bound form of ADP-ribosylation factor-6, a plasma membrane-specific regulator of endocytic membrane trafficking, and with ADP-ribosylation factor nucleotide binding site opener, an ADP-ribosylation factor guanine nucleotide exchange factor (9). Arrestin-2 also interacts with N-ethylmaleimide-sensitive fusion protein (NSF), an ATPase that binds to SNARE complexes and plays an essential role in membrane trafficking (10). The interaction of nonvisual arrestins with various proteins involved in protein trafficking supports an important functional role for arrestins in receptor internalization.

Recently, arrestins have been shown to play a role not only in “negative” regulation of GPCRs but also in transmitting “positive” signals from receptors via interaction of arrestins with Src family tyrosine kinases and mitogen-activated protein kinases such as extracellular signal-regulated kinases 1 and 2 and c-Jun N-terminal kinase 3 (11–17). In the case of extracellular signal-regulated kinases 1 and 2 and c-Jun N-terminal kinase 3 interaction, arrestin-3 also directly binds the mitogen-activated protein kinase kinase kinase Raf-1 and ASK1, respectively, thereby serving as a scaffolding molecule and mediating GPCR-stimulated activation of the specific mitogen-activated protein kinase cascade (15, 16).

Despite numerous studies suggesting an essential role of arrestins in GPCR regulation, the detailed mechanism under-
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**Experimental Procedures**

**Plasmid Constructs**—pcDNA3-FLAG-β2AR, pcDNA3-arr2, or pcDNA3-arr3 constructs were incubated in phosphate-free DMEM (Invitrogen) for 30 min and labeled for 2 h in the same medium containing 0.1 μCi/ml [32P]orthophosphate (PerkinElmer Life Sciences). For β2AR stimulation, 10 μM (-)isoproterenol was added to the labeling medium 15 min before the end of labeling. Cells were then washed with ice-cold Tris-buffered saline (TBS) and harvested for immunoprecipitation of arrestins.

**Subcellular Fractionation**—COS-1 cells grown on 60-mm dishes, labeled with [32P]orthophosphate, and incubated with or without isoproterenol were washed with TBS twice, incubated with 250 μg/ml concanavalin A in TBS on ice for 20 min, and scraped into 3 ml of hypotonic buffer (20 mM Tris, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and a mixture of protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml benzamidine, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) Cells were then lysed using a Dounce homogenizer (15 strokes), and unbroken cells and nuclei were removed by centrifugation at 1000 x g for 10 min. A crude plasma membrane fraction was prepared by centrifugation of the cell homogenate at 3000 x g for 15 min, and the pellet was then suspended in 1 ml of immunoprecipitation buffer (IP buffer) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, and protease inhibitors). The 3000 x g supernatant was then centrifuged at 300,000 x g for 30 min. The resulting pellet (vesicle membrane fraction) was dissolved in 1 ml of IP buffer. The remaining supernatant (cytosolic fraction) was diluted with an equal volume of IP buffer containing 2% Triton X-100. The protein concentration of the three subcellular fractions was determined using BCA protein assay kit (Pierce).

**Immunoprecipitation of Radiolabeled Arrestins and Autoradiography**—COS-1 cells labeled with [32P]orthophosphate were washed with ice-cold TBS twice and lysed in 1 ml of IP buffer. Insoluble proteins were removed by centrifugation at 55,000 x g for 20 min. The supernatant or various subcellular fractions were precleared by incubation with protein A-agarose for 1 h at 4 °C. Arrestin-2 or -3 proteins were immunoprecipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose for an additional 2 h. KEE is a highly specific anti-arrestin-2 antibody generated against the C-terminal 16 amino acids of arrestin-2 (KEEEDQGSPRLNDR) (24). 182 was generated against a glutathione S-transferase (GST) fusion protein containing amino acids 331-347 of arrestin-2 (GST-Arr22247-230). GST-Arr22247-230 was generated using a glutathione-S-transferase (GST)-expression vector and the GST fusion protein was purified using a glutathione-Sepharose column. KEE and 182 were then used for immunoprecipitation of arrestins. The immunoprecipitates were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose. The precipitated proteins were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose. The precipitated proteins were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose. The precipitated proteins were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose. The precipitated proteins were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose. The precipitated proteins were then washed with ice-cold TBS and dissolved in 1 ml of IP buffer. Insoluble proteins were then precipitated by incubating with purified polyclonal antibodies, KEE and 182, respectively, for 2 h and subsequently with protein A-agarose.
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![Fig. 2. Arrestin-3 phosphorylation at Thr-382. Arrestin-3, arr3-(201-409), and arr3-(284-409) (a) or arrestin-3, arr3-(1-360), and arr3-(1-370) (b) were transiently expressed in COS-1 cells, metabolically labeled with [32P]orthophosphate, immunoprecipitated, electrophoresed, and detected by autoradiography as described under “Experimental Procedures.” The expected sizes of various arrestins are indicated with arrows. Arrestin-3, arr3-(201-409), and arr3-(284-409) are phosphorylated, whereas arr3-(1-360) and arr3-(1-370) are not. c, arrestin-3, arr3(S37A), arr3(S360A,T365A), and arr3(T382A) were overexpressed in COS-1 cells, and the phosphorylation status was assessed as described above. d, alignment of C-terminal domains of arrestin-2 and -3. Phosphorylated residues are underlined and in boldface type; known clathrin binding residues are marked with plus signs; and the reported β-adaptin binding residues are denoted with asterisks. WT, wild type; WB, Western blot.]

Confocal Microscopy—Visualization of arr3-GFP fusion protein was performed as previously described (26). Briefly, HEK293 cells containing stably expressed βAR and transiently expressed arr3-GFP, arr3(T382V)-GFP, or arr3(T382E)-GFP were plated on 35-mm glass-bottomed culture dishes. Two hours before analysis, the culture medium was replaced with serum-free minimal essential medium supplemented with 10 mM HEPES. Confocal microscopy was performed on a Zeiss laser-scanning confocal microscope (LM-510) using a heated (37 °C) microscope stage as described (27).

Internalization Assay—Internalization of FLAG-βAR was assessed by enzyme-linked immunosorbent assay as described (28). Briefly, COS-1 cells transfected with pcDNA3-FLAG-βAR and various arrestin constructs were split into poly-L-lysine-coated 24-well tissue culture plates after 24 h. The next day (48 h post-transfection), cells were treated with 10 μM (-)-isoproterenol and 0.1 mM ascorbate at 37 °C for 0, 2, 5, 10, 20, or 30 min, fixed with 3.7% formaldehyde in TBS for 5 min at ambient temperature, and washed three times with TBS. Cells were then blocked with TBS containing 1% BSA (TBS/BSA) for 45 min, incubated with a primary antibody (M1, Sigma), 1:1000 dilution in TBS/BSA) for 1 h, washed three times with TBS, blocked again with TBS/BSA for 15 min, incubated with a secondary antibody (goat anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad), 1:1000 dilution in TBS/BSA) for 1 h at room temperature, and washed three times with TBS. Colorimetric visualization of antibody binding was performed using an alkaline phosphatase substrate kit (Bio-Rad), and samples were read at 405 nm in a microplate reader using Microplate Manager software (Bio-Rad). The reading from cells that did not express flag-βAR was used as blank and subtracted. The percentage of surface receptor loss was determined by calculating the change of antibody-accessible FLAG-βAR.

In Vitro Translation of Arrestin-3 and Clathrin Binding Assay—Arrestin-3 constructs were transcribed and translated in vitro using the TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer’s recommendations. Briefly, pcDNA3-arr3 (wild type, T382A, or T382C) were added to a reaction mixture containing TNT buffer, 1 μl of T7 RNA polymerase, 1 μM amino acid mixture (without leucine), 1 μl of RNasin, 25 μl of rabbit reticulocyte lysate, and 5 μl of [3H]leucine (1 mCi/ml, 170 Ci/mmol; PerkinElmer Life Sciences) in a total volume of 50 μl. The reaction was incubated for 90 min at 30 °C and stopped by snap-freezing. To calculate incorporation radioactivity, 1 μl of reaction mix was spotted onto Whatman filter paper and then successively incubated in ice-cold 10% (v/v) trichloroacetic acid for 10 min, boiling 5% (v/v) trichloroacetic acid for 10 min, and 100% ethanol for 1 min. The precipitated materials were dissolved in 1% SDS and...
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**In vitro phosphorylation of arrestin-3 by casein kinase II.** a, phosphorylation of arrestin-3 by various protein kinases. One microgram (~1 μM) of purified arrestin-3 was incubated with 0.1 μM CKI, CKII, GRK2, or GRK5 in 20 μl of buffer containing [γ-32P]ATP for 30 min at 30 °C. The reaction was stopped with SDS sample buffer, the samples were electrophoresed, and arrestin-3 phosphorylation was detected by autoradiography (upper panel). Arrestin-3 was phosphorylated only by CKII (marked with a filled arrowhead). Autophosphorylated kinase bands are marked with open arrows. Protein staining shows that the same amount of arrestin-3 was loaded in each lane (lower panel). b, 1 μg of purified arrestin-2 or -3 was incubated with 0.1 μM CKII in 20 μl of buffer containing [γ-32P]ATP at 30 °C for the indicated times. Reactions were stopped by the addition of SDS sample buffer, the samples were electrophoresed, and the arrestin bands were excised and counted. The data represent the means ± S.E. of three independent experiments. c, COS-1 cell lysates containing expressed arrestin-3 or arr3(T382A) were incubated with or without 0.05 μM CKII for 1 h at 30 °C. Reactions were stopped on ice, the arrestins were immunoprecipitated and electrophoresed, and the phosphorylation was determined by autoradiography. Whereas arrestin-3 phosphorylation increased in the presence of CKII, no phosphorylation of arr3(T382A) was detected (upper panel). Western blot analysis showed that a similar amount of arrestin was loaded in each lane (lower panel).

50 mM NaOH, and radioactivity was counted in a liquid scintillation counter with scintillant. The in vitro translated proteins were also analyzed by SDS-PAGE.

**Fig. 4.** β2AR stimulation promotes arrestin-3 dephosphorylation. a, COS-1 cells expressing either arrestin-3 or arrestin-3 and β2AR were metabolically labeled with [32P]orthophosphate for 2 h and then incubated with (+) or without (−) 10 μM isoproterenol (ISO) for 15 min at 37 °C. Crude plasma membrane (PM), vesicle membrane (VM), and cytosol fractions were then prepared by differential centrifugation. Arrestin-3 phosphorylation in the total cell lysate and each fraction was determined as described under “Experimental Procedures.” b, arrestin-3 and FLAG-β2AR were transiently expressed in COS-1 cells. At 48 h post-transfection, cells were incubated with (+) or without (−) 10 μM isoproterenol for 2 min at 37 °C. The cells were then lysed, and arrestin was immunoprecipitated using an arrestin-3-specific polyclonal antibody as described under “Experimental Procedures.” Co-immunoprecipitated PP2A was detected using a monoclonal antibody against the catalytic subunit of PP2A (PP2A-C) (upper panel), whereas immunoprecipitated arrestin-3 was detected using the monoclonal antibody PAC1 (lower panel). Endogenous PP2A-C in the lysate is shown in the upper panel.

The clathrin cage binding assay has been described previously (7). Briefly, 0.5 nm of in vitro synthesized [3H]-labeled arrestins were incubated with or without 200 nM clathrin cages for 10 min at ambient temperature in a total volume of 50 μl in 100 mM Na-MES, pH 6.8, 1 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μg/ml benzamidine. Samples were then cooled on ice, loaded onto a 75-μl cushion of 0.2 M sucrose in 100 mM Na-MES, pH 6.8, and centrifuged at 100,000 rpm in a TLA100.1 rotor for 5 min at 4 °C. Pellets were solubilized in 25 μl of SDS-sample buffer supplemented with 100 mM Tris-HCl, pH 8, heated at 100 °C for 5 min, and run on 10% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue, destained, incubated with ENTTENIFY (PerkinElmer Life Sciences), dried, and exposed to x-ray film. After development, films were scanned with a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) to quantitate the amount of radioactively labeled arrestins that were co-sedimented with clathrin cages. The percentage of bound arrestin was calculated by comparing the signal from the co-sedimented arrestins to a set of arrestin standards. Binding of in vitro translated arrestins to clathrin-coated vesicles (CVs) was done in the same manner as the clathrin cage binding assay using 50 nm CVs (50 nm clathrin trimers). Clathrin cages and CVs were generously provided by Dr. James H. Keen (Thomas Jefferson University).

**In Vitro Binding of Arrestins to GST-β2-Adaptin—GST or GST-β2-adaptin (residues 700–937) fusion proteins were expressed in BL21(De3)pLysS cells and purified on glutathione-agarose beads. Five micrograms of purified GST fusion proteins were incubated with 10 μg of COS-1 cell lysates containing overexpressed arrestin-3, arr3(T382V), or arr3(T382E) in binding buffer (20 mM HEPES, pH 7.2, 120 mM...**

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were quantified by determining band densities using a densitometer. Arrestins were detected by fluorography. Levels of radioactive arrestin and electrophoresed on a 10% SDS-polyacrylamide gel, and radioactive

cated chase period (8, 16, and 32 h). Arrestins were immunoprecipitated

serum and harvested either immediately (0 h chase) or after the indi-

cells were washed twice with DMEM containing 10% fetal bovine

lysed in 1 ml of coimmunoprecipitation buffer containing 20 mM Tris-

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potassium acetate, 0.1 mM dithiothreitol, 0.2% Triton X-100) for 1 h at 4

°

in a total volume of 100 μl). The beads were then washed twice

with 500 μl of binding buffer, and bound arrestins were eluted by

boiling the beads in SDS sample buffer for 5 min. The samples were

electrophoresed on a 10% polyacrylamide gel, and radioactive

boiling the beads in SDS sample buffer for 5 min. The samples were

electrophoresed on a 10% polyacrylamide gel, and transferred to a nitrocel-

immunoprecipitation buffer after 48 h, and arrestin-3 was immunoprecipi-

cation at 55,000

°

M isoproterenol. All three forms of arr3-

°

M (100-, 200-, and 300-s) treatment with 10

°

A phosphoprotein that migrated to a position similar to arres-

wti

Search for Phosphorylation-dependent Arrestin-3-binding Proteins—

Wild type arrestin-3, arr3(WT)-GFP, and arr3(T382E) were expressed in

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wti

RESULTS AND DISCUSSION

Constitutive Phosphorylation of Arrestin-3 in COS-1 Cells—

Previous studies have shown that arrestin-2 is constitutively

phosphorylated in HEK293 cells and that the dephosphoryla-

tion of arrestin-2 that occurs upon stimulation of β AR is important

for receptor internalization (20). To test whether arrestin-3 is also a

phosphoprotein, COS-1 cells transiently expressing arrestin-2 or -3 were

metabolically labeled with [35S]methionine and [35S]cysteine by incubating in Met/Cys-free DMEM containing 0.2 mCi/ml of Trans-35S-LABEL (ICN) for 2 h at 37 °C (pulse). Cells were then washed twice with DMEM containing 10% fetal bovine

serum and harvested either immediately (0 h chase) or after the indi-

cated chase period (8, 16, and 32 h). Arrestins were immunoprecipitated

and electrophoresed on a 10% SDS-polyacrylamide gel, and radioactive

arrestins were detected by fluorography. Levels of radioactive arrestin

and electrophoresed on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. Proteins were

stained with Coomassie Blue, and arrestins were detected by probing

the membrane with monoclonal antibody F4C1.

Next, we performed phosphoamino acid analysis of the ra-

dilabeled arrestins. The arrestins were excised from the PVDF

membrane, and the proteins were subjected to acid hydrolysis. A

phosphoprotein that migrated to a position similar to arrestin-

3 (from the control cells without arrestin overexpression)

was also included in the analysis. As shown in Fig. 1b, arrest-

in-3 contained only phosphoserine as previously reported, whereas arrestin-3 had a major phosphothreonine band and a
minor phosphoserine band. A phosphoserine band was also present in the control sample (without arrestin overexpression). From subsequent mutagenesis studies and phosphoamino acid analyses (see below), we conclude that arrestin-3 is mainly phosphorylated on threonine.

Mapping of Phosphorylation Sites in Arrestin-3—To identify the specific residues phosphorylated in arrestin-3, we first examined the phosphorylation of various truncated arrestin-3 proteins. Amino- or carboxyl-terminally truncated proteins were expressed in COS-1 cells, and their phosphorylation was compared with wild type arrestin-3 by metabolic labeling and immunoprecipitation. Amino-terminally truncated proteins, arr3(201–409) and arr3(284–409), were phosphorylated similar to wild type arrestin-3, suggesting that the phosphorylation sites reside after residue 284 (Fig. 2a). Analysis of the carboxyl-terminally truncated proteins arr3(1–360) and arr3(1–370) suggested that the phosphorylation occurs from residue 370 to 409, since neither of these proteins was phosphorylated (Fig. 2b). All of the truncated proteins were expressed and immunoprecipitated comparably with wild type protein except for arr3(1–360) that was immunoprecipitated less well (data not shown).

We next made several point mutations where threonine and serine residues in the carboxyl terminus of arrestin-3 were changed to alanine. As shown in Fig. 2c, mutation of Thr-382 to alanine (T382A) completely abolished arrestin-3 phosphorylation, whereas mutation of Ser-337 (S337A) or Ser-360 and Thr-365 (S360A/T365A) were still phosphorylated, albeit to a reduced level. Therefore, we believe that the constitutive phosphorylation of arrestin-3 occurs primarily at Thr-382. It is still possible, however, that there is additional phosphorylation on serine or threonine residues of arrestin-3 that might occur after the primary phosphorylation of Thr-382.

Arrestin-3 Is Phosphorylated by CKII—To identify the protein kinase responsible for arrestin-3 phosphorylation, we initially performed in vitro kinase assays using purified recombinant arrestin-3 and various purified protein kinases. Since Thr-382 is in an acid-rich region of arrestin-3 (382TDDD; Fig. 2d), we tested whether arrestin-3 was a substrate for various acidotropic kinases. These included CKII and GRK2, both of which prefer substrates that contain acidic residues amino-terminal to the phosphorylation site (29, 30), and CKII, which has a consensus sequence of (S/T)XX(D/E) (31). We also tested GRK5 since one would predict that GRKs and arrestins are in close proximity when associated with GPCRs. These studies revealed that arrestin-3 is effectively phosphorylated by CKII but is not a substrate for CKI, GRK2, or GRK5 (Fig. 3a). Additional analysis showed that GRK2 and GRK5 were also unable to phosphorylate arrestin-3 in the presence of phospholipids or light-activated rhodopsin, two GRK activators (data not shown) (32, 33). CKII seems to phosphorylate a single residue, because arrestin-3 phosphorylation by CKII was saturable over time with a stoichiometry of ~0.5 mol of phosphate/mol of arrestin-3 (Fig. 3b). In contrast, arrestin-2 was not significantly phosphorylated by CKII.

To test whether CKII phosphorylates Thr-382, we prepared lysates from COS-1 cells overexpressing wild type or T382A arrestin-3 and performed in vitro kinase assays in the presence or absence of purified CKII. Arrestins were then immunoprecipitated, and the phosphorylation was analyzed by SDS-PAGE and autoradiography. Wild type arrestin-3 was effectively phosphorylated by exogenous CKII, although there was some phosphorylation in the absence of additional kinase (possibly by endogenous CKII) (Fig. 3c). In contrast, the T382A mutant was not phosphorylated by either the endogenous kinases or by recombinant CKII. Phosphorylation of Thr-382 by CKII was also confirmed using purified glutathione S-transferase fusion proteins of arrestin-3 as substrates. While CKII efficiently phosphorylated GST-arr3-(330–409), it failed to phosphorylate a T382A mutant of GST-arr3-(330–409) (data not shown).

Taken together, these results demonstrate that CKII phosphorylates Thr-382 and is probably the kinase responsible for arrestin-3 phosphorylation in intact cells.

Arrestin-3 Is Dephosphorylated upon β2-Adrenergic Receptor Stimulation—To gain further insight into arrestin-3 phosphorylation, we examined the phosphorylation status of arrestin-3 during GPCR stimulation. COS-1 cells expressing arrestin-3 and β2AR were metabolically labeled with [32P]orthophosphate and treated with or without the β2-agonist isoproterenol for 15 min, and the phosphorylation of arrestin-3 was determined by immunoprecipitation, SDS-PAGE, and autoradiography. Initial analysis revealed that arrestin-3 phosphorylation decreased ~35% after β2-agonist treatment, suggesting that arrestin-3 is dephosphorylated upon activation of β2AR (Fig. 4a, upper panel).

Arrestins are cytosolic proteins that translocate to the plasma membrane upon GPCR activation and subsequent phosphorylation. Dependent on the receptor subtype, nonvisual arrestins can then traffic with the receptor initially to clathrin-coated pits and in some cases to endocytic vesicles (26).
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Arr3(T382V) to mimic the dephosphorylated form of arrestin-3, and we generated arr3(T382E) to mimic phosphorylated arrestin-3. To study arrestin localization, we generated fusion proteins in which enhanced green fluorescent protein was fused to the carboxyl terminus of arr3, arr3(T382V), and arr3(T382E). The various arr3-GFP fusion proteins were then expressed in HEK293 cells that had been stably transfected with β2AR and visualized using time lapse confocal microscopy. Before receptor activation, wild type and mutant arrestins were found exclusively in the cytosol (Fig. 5, time 0), suggesting that phosphorylation does not regulate the basal intracellular localization of arrestin-3. We next studied the translocation of arrestin-3 after β2AR stimulation, an approach proven useful for investigation of real time arrestin translocation and modeling of arrestin/receptor interaction (36). As shown in Fig. 5, α-2AR cellulose, and detected with purified polyclonal antibody against arrestin-3. In vitro synthesized tritiated arrestin-3, arr3(T382A) or arr3(T382E) were transiently expressed in COS-1 cells. At 48 h post-transfection, cells were treated with (+) or without (−) 10 μM isoproterenol for 5 min at 37 °C. Arrestins were immunoprecipitated from cell lysates and co-immunoprecipitated Src was detected with a monoclonal antibody as described under “Experimental Procedures” (upper panel). Expression of Src was determined by immunoblotting cell lysates with a Src monoclonal antibody (lower panel).

Because of the dynamics of arrestin localization, we next examined the phosphorylation status of arrestin-3 in various intracellular compartments. After metabolic labeling and stimulation of β2AR, COS-1 cells were lysed and fractionated by differential centrifugation. Agonist-dependent dephosphorylation of arrestin-3 was significant in the plasma membrane fraction, whereas the extent of arrestin-3 phosphorylation in the vesicle membrane and cytosolic fractions remained largely unchanged (Fig. 4a). This suggests that dephosphorylation of arrestin-3 mainly occurs at the plasma membrane or that dephosphorylated arrestin-3 is preferentially recruited to the plasma membrane. It is noteworthy that significant arrestin-3 dephosphorylation also occurred in cells with endogenous β2AR, suggesting that this process is highly efficient. Although the detailed mechanism of arrestin-3 dephosphorylation is unknown, it is interesting to note that the catalytic subunit of endogenous PP2A coimmunoprecipitates with expressed arrestin-3 in COS-1 cells (Fig. 4b). Previous studies implicated PP2A in β2AR dephosphorylation, and it was present in a multicomponent complex containing β2AR, protein kinase A, PP2B, and gravin (A kinase anchoring protein 250) (34, 35).

Phosphorylation of Arrestin-3 Does Not Regulate Its Translocation to the Plasma Membrane after β2AR Stimulation—To investigate the potential functional role of arrestin-3 phosphorylation, we generated the mutant constructs arr3(T382A) and arr3(T382V) to mimic the dephosphorylated form of arrestin-3, and we generated arr3(T382E) to mimic phosphorylated arrestin-3. To study arrestin localization, we generated fusion proteins in which enhanced green fluorescent protein was fused to the carboxyl terminus of arr3, arr3(T382V), and arr3(T382E). The various arr3-GFP fusion proteins were then expressed in HEK293 cells that had been stably transfected with β2AR and visualized using time lapse confocal microscopy. Before receptor activation, wild type and mutant arrestins were found exclusively in the cytosol (Fig. 5, time 0), suggesting that phosphorylation does not regulate the basal intracellular localization of arrestin-3. We next studied the translocation of arrestin-3 after β2AR stimulation, an approach proven useful for investigation of real time arrestin translocation and modeling of arrestin/receptor interaction (36). As shown in Fig. 5, α-2AR...
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stabilization results in rapid translocation of arr3, arr3(T382V), and arr3(T382E) from the cytosol to the plasma membrane. Quantification reveals that the initial rates of translocation were similar for arr3, arr3(T382V), and arr3(T382E) and suggests that the rate of receptor association is not regulated by arrestin-3 phosphorylation. These results are similar to arrestin-2, where the phosphorylation of Ser-412 by extracellular signal-regulated kinases 1 and 2 appears to have no effect on receptor interaction (36). Our results also suggest that the decrease in phosphorylated arrestin-3 that occurs upon β2AR stimulation (Fig. 4) is probably due to dephosphorylation at the plasma membrane rather than the preferential translocation of the dephosphorylated protein to the plasma membrane.

Internalization and Recycling of β2AR Is Not Regulated by Arrestin-3 Phosphorylation—Because β2AR stimulation promotes arrestin-3 dephosphorylation, we hypothesized that the phosphorylation state of arrestin-3 might influence its ability to regulate β2AR function. Nonvisual arrestins mediate internalization of many GPCRs by concomitant binding to receptors and components of the endocytic machinery including clathrin and β2-adaptin (7, 8). Therefore, we tested whether agonist-promoted β2AR internalization was regulated by the phosphorylation state of arrestin-3. In COS-1 cells, the rate and extent of FLAG-β2AR internalization was significantly decreased by overexpression of wild type arrestin-3 (Fig. 6a). Additional studies that varied the expression level of arr3, arr3(T382A), and arr3(T382E) also did not detect differences in their ability to promote β2AR internalization (not shown). Similarly, arr3, arr3(T382A), arr3(T382V), and arr3(T382E) promoted internalization of β2AR(Y326A), a mutant receptor partially impaired in agonist-promoted phosphorylation and internalization (37, 38), to a similar extent (not shown). Moreover, arr3, arr3(T382A), and arr3(T382E) all promoted a similar level of FLAG-β2AR internalization as assessed by immunofluorescence microscopy (not shown). Taken together, these results suggest that the phosphorylation state of arrestin-3 does not significantly alter its ability to promote β2AR internalization. These findings are in contrast to arrestin-2, where previous studies found that arr2(S412D), which mimics the phosphorylated form of arrestin-2, functioned as a dominant negative mutant and inhibited β2AR internalization. This defect of arr2(S412D) has been ascribed to an inability to interact with clathrin (20).

Via its ability to promote β2AR internalization, arrestin-3 also promotes subsequent resensitization and recycling of β2AR (39). Thus, we next tested whether the phosphorylation state of arrestin-3 influences β2AR recycling. COS-1 cells expressing arr3, arr3(T382A), or arr3(T382E) along with FLAG-β2AR were stimulated with isoproterenol for 30 min, washed extensively with media, and treated with the β-antagonist alprenolol for 90 min to allow internalized receptors to recycle back to the plasma membrane. The profiles of FLAG-β2AR internalization and recycling in the presence of the various forms of arrestin-3 were indistinguishable (Fig. 6b). Therefore, internalization and recycling of the β2AR do not appear to be regulated by arrestin-3 phosphorylation.

Phosphorylation of Arrestin-3 Does Not Regulate Interaction with Clathrin or β-Adaptin—Although we did not see a defect in the ability of arr3(T382E) to promote β2AR internalization, we were interested in determining whether arrestin-3 phosphorylation directly affects clathrin interaction, since Thr-382 is close to the primary clathrin binding site in arrestin-3 (Fig. 2d). This was tested using an established in vitro clathrin binding assay (7). Radiolabeled arrestins were prepared by in vitro translation and incubated with purified clathrin cages, and clathrin-bound arrestins were pelleted by centrifugation, resolved by SDS-PAGE, and detected by fluorography. As shown in Fig. 7a, arr3, arr3(T382A), and arr3(T382E) bound to clathrin cages to a similar extent. We also analyzed the ability of arr3, arr3(T382A), and arr3(T382E) expressed in COS-1 cells to bind to purified GST-clathrin terminal domain and found a
comparable level of binding (data not shown). Taken together, these results suggest that phosphorylation of arrestin-3 does not directly influence its interaction with clathrin.

Since nonvisual arrestins also interact with the β-adaptin subunit of the adaptor protein AP2 (8), we next investigated whether phosphorylation of arrestin-3 affects its interaction with β-adaptin. This was assessed by co-expressing arrestins and β2ARs in COS-1 cells, treating the cells with or without β-agonist for 2 min, and analyzing arrestin/β-adaptin interaction by immunoprecipitating the arrestin and blotting for endogenous β-adaptin. As shown in Fig. 7b, arrestin-binding to β-adaptin is detected in unstimulated cells but is increased ~2-fold by receptor stimulation. Arr3(T382A) and arr3(T382E) bind β-adaptin comparably with wild type arrestin-3, suggesting that the interaction of these proteins is not regulated by arrestin-3 phosphorylation. Similarly, we found that arr3, arr3(T382A), and arr3(T382E) expressed in COS-1 cells bound comparably with purified GST-β2-adaptin appendage domain (Fig. 7c). To confirm these results, we also analyzed interaction of arrestin-3 with CVs. CVs contain clathrin and AP2 and thus represent a more physiological model for clathrin-coated pits.

Similar to the clathrin cage binding assay, arr3, arr3(T382A), and arr3(T382E) bound equally well to CVs (Fig. 7d). Overall, these results suggest that arrestin-3 phosphorylation at Thr-382 does not directly regulate interaction with either clathrin or β-adaptin.

Interaction between Arrestin-3 and Src Is Independent of Arrestin-3 Phosphorylation—Recently, a role for arrestins as positive transducers of GPCR signaling has been appreciated (40). Arrestins can interact with Src family members (11, 14, 17) and function as scaffolds in GPCR-mediated activation of mitogen-activated protein kinases (12, 13, 15, 16). Interestingly, the interaction between Src and arrestin-2 was predicted to be regulated by the phosphorylation state of arrestin, since arr2(S412D) did not bind Src (11). Accordingly, we tested whether the phosphorylation state of arrestin-3 would regulate Src interaction. Arrestins were co-expressed with Src and β2ARs in COS-1 cells, β2ARs were stimulated with agonist for 5 min to promote arrestin/Src interaction, and the interaction was then assessed by immunoprecipitating the arrestin and blotting for Src. Src was found to coprecipitate with arr3, arr3(T382A), arr3(T382V), and arr3(T382E) to a comparable extent, suggesting that the phosphorylation of arrestin-3 does not affect Src interaction (Fig. 8).

Cellular Degradation of Arrestin-3 Is Not Regulated by Its Phosphorylation—Recently, arrestin-3 has been reported to be ubiquitinated by the E3 ubiquitin ligase Mdm2 (41). Because ubiquitin-mediated degradation is often regulated by the phosphorylation state of the target protein (42, 43), we next evaluated the half-life of the various arrestin-3 mutants by pulse-chase labeling. Arrestins were expressed in COS-1 cells, labeled with [35S]methionine and [35S]cysteine for 2 h, and then immunoprecipitated following a chase with nonradioactive media. The half-lives of arrestin-3, arr3(T382A), and arr3(T382E) were all ~13.5 h (Fig. 9). Therefore, degradation of arrestin-3 is not regulated by phosphorylation at Thr-382.

The Phosphorylation of Arrestin-3 Modulates Formation of a Protein Complex—Since our studies suggest that arrestin-3 phosphorylation does not regulate its interaction with several known binding partners including clathrin, β-adaptin, and Src, we decided to take a more general approach to identify a target interaction. This involved expressing arr3, arr3(T382V), and arr3(T382E) in HEK293 cells and immunoprecipitating the arrestin using a purified anti-arrestin-3 polyclonal antibody bound to protein A-conjugated agarose beads. The beads were washed and incubated in SDS sample buffer for 2 h at 27 °C, and the bound proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and stained with Coomassie Blue. As seen in the upper panel of Fig. 10, protein staining detected the ~50-kDa arrestin-3 proteins as well as the ~100-kDa IgG heavy chain dimers. In addition, we noted some protein staining near the top of the gel (marked with a question mark) that was consistently most prominent in the arr3(T382V) lane, less in the wild type arrestin-3 lane, and least prominent in the arr3(T382E) lane. We hypothesize that this band represents a protein or proteins that selectively interact with dephosphorylated arrestin-3. Using a lower percentage gel, the size of the band(s) was estimated to be >300 kDa.

Interestingly, the high molecular weight band(s) were also detected by immunoblotting with an anti-arrestin monoclonal antibody (Fig. 10, lower panel). Similar to the protein staining, arrestin-3 was most prominent in the arr3(T382V) lane and was not evident in the arr3(T382E) sample. Therefore, we believe that the high molecular weight band represents a multiprotein complex consisting of dephosphorylated arrestin-3 and unidentified arrestin-3 binding protein(s). The reason why the individual protein components of the complex were not visualized with treatment of SDS and β-mercaptoethanol in the SDS-sample buffer is not clear. However, there are examples where protein complexes such as oligomericGPCRs are not resolved by denaturing SDS-PAGE (44–46).

In summary, we found that arrestin-3 is constitutively phosphorylated at Thr-382 by casein kinase II in COS-1 cells and becomes dephosphorylated after stimulation of β2AR. The phosphorylation state of arrestin-3 does not appear to regulate interaction with β2AR, clathrin, β-adaptin, or Src. Rather, it seems to regulate interaction with an unidentified arrestin-3-binding protein. Differences between the regulatory role of arrestin-2 and -3 phosphorylation may contribute to the different cellular functions of these highly homologous proteins in GPCR signaling and regulation.

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