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Article

Arrestin2/Clathrin Interaction Is Regulated by Key N- and C-Terminal Regions in Arrestin2[†]

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ABSTRACT: The interaction of nonvisual arrestins with clathrin is an important step in mediating the endocytosis of cell surface receptors. Previous studies have shown that mutation of the clathrin-binding box in arrestin leads to severe defects in arrestin-mediated trafficking. However, little is known about how arrestin/clathrin interaction is regulated. Here we show that both the N- and C-terminal regions of arrestin2 function to inhibit basal interaction with clathrin. Truncation analysis revealed that clathrin binding increases as the C-tail of arrestin2 is shortened while site-directed mutagenesis identified Glu-404, Glu-405, and Glu-406 as being primarily responsible for this inhibition. Mutagenesis also identified Lys-4, Arg-7, Lys-10, and Lys-11 within the N-terminus as playing a key role in regulating clathrin binding. Based on similarities with visual arrestin, Lys-10 and Lys-11 likely function as phospho sensors in arrestin2 to initially discriminate the phosphorylation status of target receptors. Analysis of the arrestin2 structure reveals that Arg-7, Lys-10, and Lys-11 are in close proximity to Glu-389 and Asp-390, suggesting that these residues may form intramolecular interactions. In fact, simultaneous mutation of Glu-389 and Asp-390 also leads to enhanced clathrin binding. These results reveal that multiple intramolecular interactions coordinately regulate arrestin2 interaction with clathrin, highlighting this interaction as a critical step in regulating receptor trafficking.

G protein-coupled receptors (GPCRs)[1] represent the largest family of cell surface signaling molecules and the most common target of pharmaceutical drugs (*1*). These receptors regulate the activity of a number of effector molecules and control many biological functions including cell growth, differentiation, angiogenesis, neurotransmission, sensory perception, and cardiovascular function (*2*). In order for proper signaling to be achieved, these receptors must be tightly regulated. Three primary modes of regulation control GPCRs: (1) desensitization, in which the receptor loses its ability to signal in the presence of stimuli, (2) internalization, which lowers the number of receptors on the cell surface, and (3) degradation, which lowers the total level of receptors inside the cell (*3*). Upon ligand binding, G protein-coupled receptor kinases (GRKs) phosphorylate specific serine and threonine residues within the intracellular regions of the receptor, promoting the recruitment and high-affinity binding of arrestin (*3*). Arrestins can be divided into two classes, visual and nonvisual, based on their function and localization. The nonvisual arrestins, which include arrestin2 and arrestin3 (also referred to as β-arrestin1 and β-arrestin2, respectively), have been shown to regulate GPCR desensitization and trafficking as well as G protein independent signaling (*3*−*5*).

The ability of the nonvisual arrestins to regulate endocytosis was first described for the β_2-adrenergic receptor (β_2AR) (*6*, *7*). Fluorescence microscopy revealed that overexpression of arrestin2 leads to a diffuse localization throughout the cytoplasm and nucleus of the cell (*7*). Interestingly, activation of the β_2AR promoted the redistribution of arrestin2 to various puncta within the cell and resulted in the receptor, arrestin, clathrin, and the clathrin adaptor protein AP-2 colocalizing at the plasma membrane (*7*). These results suggest that the nonvisual arrestins are recruited into clathrin-coated pits following receptor binding. In fact, binding to the activated phosphorylated receptor appears to promote arrestin binding to two key endocytic proteins, clathrin and AP-2 (*7*, *8*). The nonvisual arrestins interact directly with clathrin through an LϕEϕ(D/E) motif, also referred to as the clathrin-binding box, located within the C-terminal tail of arrestin (*7*, *9*). Deletion of this motif in arrestin2 or mutation of the motif in arrestin3 leads to decreased clathrin binding and receptor endocytosis (*9*, *10*). The nonvisual arrestins in turn bind to residues 89−100 of the clathrin terminal domain (*11*). Although direct binding studies have defined the interaction between arrestin2 and clathrin, little is known about how this interaction is regulated in the basal state of arrestin. For example, under basal conditions, full-length arrestin2 is diffusely localized throughout the cytoplasm while a construct containing the C-terminal domain of arrestin2 (residues 319−418) constitutively localizes in clathrin-coated pits (*12*). These results suggest that the

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[1]Abbreviations: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; β_2AR, β_2-adrenergic receptor; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, AP-2, adaptor protein complex 2; HA, hemagglutinin.

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arrestin2 holoprotein contains determinants that inhibit clathrin binding.

Here we show that both the N- and C-terminal regions of arrestin2 contribute to regulating clathrin binding. Truncation and site-directed mutagenesis identified acidic residues within the extreme C-tail (Glu-404, Glu-405, and Glu-406) and basic residues within the N-terminus (Lys-4, Arg-7, Lys-10, and Lys-11) that play a key role in regulating clathrin binding. Interestingly, Lys-10 and Lys-11 correspond to Lys-14 and Lys-15 in visual arrestin (arrestin1), which have been shown to function as the initial phospho sensor in recognizing phosphorylated rhodopsin (*13*). These results suggest that the binding of arrestin2 to clathrin is regulated by multiple mechanisms, highlighting the importance of this interaction in the regulation of GPCR trafficking.

MATERIALS AND METHODS

Materials. A GST fusion construct containing the terminal domain of clathrin, GST-clathrin-(1−363), was kindly provided by Dr. James Keen while a GST-β2-adaptin appendage construct (residues 700−937) was a generous gift from Dr. Harvey McMahon. Antibodies were obtained from the following sources: clathrin heavy chain monoclonal, arrestin2 monoclonal, and β-adaptin monoclonal (BD Biosciences), AlexaFluor conjugated secondary antibodies (Molecular Probes), and IRDye 800 conjugated secondary antibody (Rockland). Arrestin2 and arrestin3-selective rabbit polyclonal antibodies were previously described (*14*, *15*).

Cell Culture, Transfection, and Cell Lysis. Cos 1 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 25 mM Hepes, pH 7.5, at 37 °C and 5% CO_2. Ten centimeter dishes of Cos 1 cells were transiently transfected using FuGENE 6 (Roche) following the manufacturers' recommendations. Briefly, Cos 1 cells were transfected with various amounts of wild-type or mutant arrestin2 DNA to provide similar levels of arrestin protein expression. At 48 h posttransfection, cells were rinsed with phosphate-buffered saline, and lysis buffer (20 mM Hepes, pH 7.2, 100 mM NaCl, 10 mM EDTA, 0.02% Triton X-100, and Roche protease inhibitor mixture) was added. Cells were lysed by freeze/thaw, scraped, and sonicated (10 s at 15% amplitude with 2 s bursts) on ice. Lysates were centrifuged (30000*g*, 20 min at 4 °C), aliquoted, flash frozen, and stored at −80 °C for later use in GST pulldown assays.

Arrestin2 Purification. The cDNA of bovine wild-type arrestin2, arrestin2-(1−393), and arrestin2-S412D were cloned into pTrcHisB using *Nco*I and *Hin*dIII, which results in removal of the His tag from the vector, and transformed into BL21-(DE3)pLysS cells. Each construct was purified as previously described with minor modifications (*11*, *16*).

Analysis of Arrestin Interaction with Clathrin and β2-Adaptin. GST-clathrin-(1−363) and GST-β2-adaptin-(700−937) were expressed in BL21pLysS cells, grown at 37 °C, and induced with 0.2 mM IPTG at an OD_{600} of 0.3−0.4. Following induction, cells expressing GST-clathrin-(1−363) or GST-β2-adaptin-(700−937) were grown for 3 h at 37 °C or overnight at 25 °C, respectively. Cells were pelleted and resuspended in bacterial lysis buffer (20 mM Hepes, pH 7.4, 0.2 M NaCl, 5 mM EDTA, plus Roche protease inhibitor mixture). Cells were incubated for 15 min at room temperature, and Triton X-100 was added to a final concentration of 1%. Cells were lysed by freeze/thaw and Polytron disruption and centrifuged at 40000*g* for 20 min. Lysates were aliquoted, flash frozen, and stored at −80 °C.

Glutathione-Sepharose 4B beads (Amersham) were washed three times with PBS and resuspended in an equal volume of PBS to make a 50% slurry. Cell lysates were thawed on ice and incubated with the washed beads for 1.5 h at 4 °C. The beads were washed three times in bacterial lysis buffer plus 1% Triton X-100, followed by three washes in GST bead wash buffer (20 mM Hepes, pH 7.4, 0.2 M NaCl, 1 mM EDTA).

Purified arrestins (10 pmol) or Cos 1 cell lysates overexpressing arrestin (100 μg) were incubated with GST beads in binding buffer (20 mM Hepes, pH 7.2, 120 mM potassium acetate, 0.1% Triton X-100) for 1 h at room temperature. For pulldowns using Cos 1 lysates overexpressing arrestin, binding buffer and 100 μg of Cos 1 lysate were combined and mixed, and 2.5 μL was removed and saved as "input" (1.25% of total reaction). GST beads were added, and the mixture was rocked at room temperature for 1 h. The beads were pelleted (1000 rpm, 1 min at room temperature) and washed four times with binding buffer, and bound proteins were eluted by boiling in SDS sample buffer for 5 min. Eluted proteins as well as input were separated on a 10% SDS−PAGE gel, transferred to nitrocellulose, and detected using a polyclonal arrestin2 antibody followed by an AlexaFluor 680 conjugated secondary antibody. Blots were quantitated using a Licor Odyssey scanner, and binding of the various mutants was normalized to the level of overexpressed arrestin in the input and compared to the binding of wild-type arrestin2 (set to 1).

Receptor Internalization Assay. Internalization of Flag-β_2AR was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (*10*). Briefly, Cos 1 cells were transiently transfected with 2.5 μg of Flag-β_2AR and 1 μg of various arrestin2 constructs and were split onto poly(L-lysine)-coated 24-well plates 24 h posttransfection. The following day, the cells were washed once with serum-free media and incubated in serum-free media with or without 10 μM (−)-isoproterenol and 0.1 mM ascorbic acid for various times at 37 °C. The cells were washed with Tris-buffered saline (TBS), fixed with 3.7% formaldehyde/TBS for 5 min at room temperature, and washed three times with TBS. Cells were blocked with 2.5% BSA/TBS for 45 min and incubated with M2 anti-FLAG HRP conjugated primary antibody for 2 h. The cells were washed three times with TBS, incubated with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) at room temperature, and read at 405 nm following color change using Microplate Manager software (Bio-Rad).

RESULTS

Role of the Arrestin2 C-Terminal Tail in Regulating Clathrin Binding. Under basal conditions, arrestin2 is diffusely localized throughout the cytoplasm and nucleus of the cell (*7*). A construct containing only the C-terminal tail of arrestin2 (residues 319−418), however, constitutively localizes in clathrin-coated pits (*12*). This construct was also shown to act as a dominant negative mutant and inhibit the internalization of the β_2AR, possibly through the sequestration of endogenous clathrin (*12*). These studies, however, did not analyze the binding of arrestin2-(319−418) to components of the endocytic machinery such as clathrin and AP-2. To analyze this, we overexpressed full-length arrestin2 (residues 1−418) and arrestin2-(319−418) in Cos 1 cells and analyzed the ability to bind a GST fusion protein containing the terminal domain of clathrin, GST-clathrin-(1−363), or the β-adaptin ear domain of AP-2, GST-β_2-adaptin-(700−937).

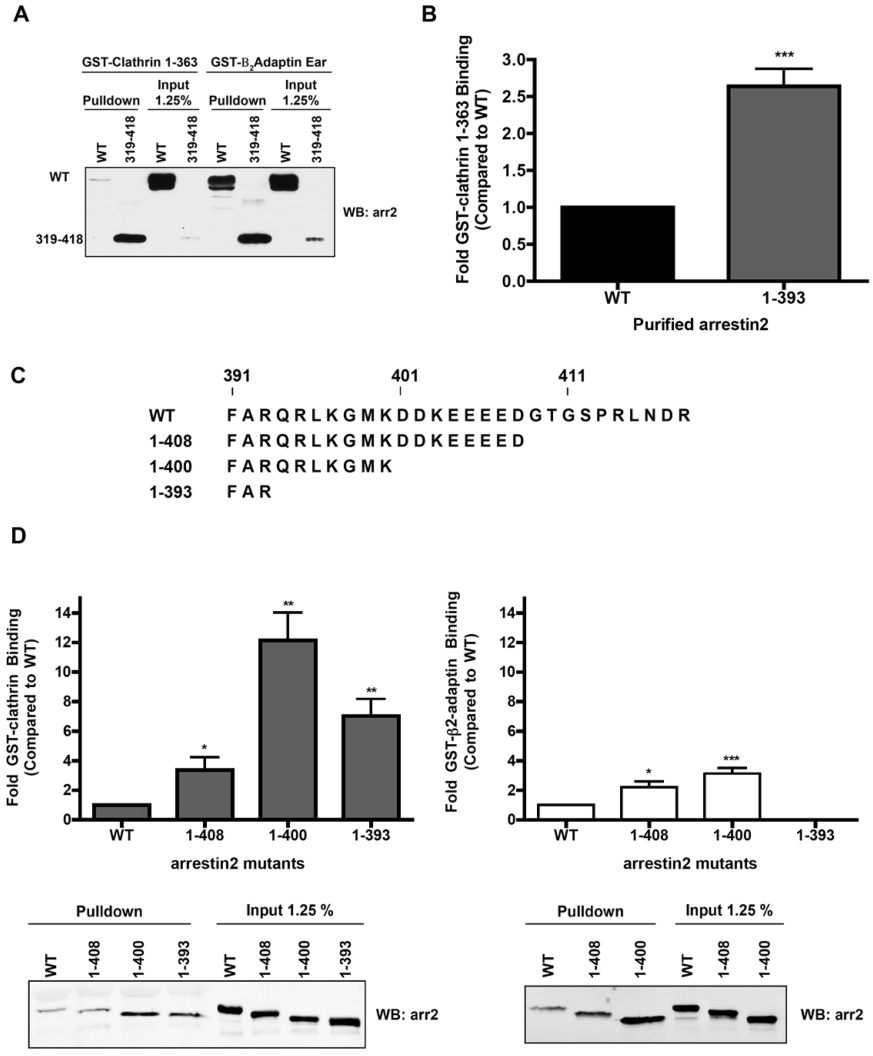

FIGURE 1: The C-terminal tail of arrestin2 regulates clathrin binding. (A) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) or 100 pmol of GST-β2-adaptin (700−937) and 100 μg of Cos 1 lysate overexpressing wild-type arrestin2 or arrestin2 319−418. (B) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 10 pmol of purified wild-type or truncated arrestin2. Bars represent the mean ± SE from ten independent experiments. (C) Sequence of the arrestin2 truncations used in the binding assays. (D) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) (left panel) or 100 pmol of GST-β_2-adaptin (700−937) (right panel) and 100 μg of Cos 1 cell lysate overexpressing wild-type or truncated arrestin2. Bound arrestins were eluted, separated on a 10% SDS−PAGE gel, transferred to nitrocellulose, and probed with an arrestin polyclonal antibody, followed by an AlexaFluor 680 conjugated secondary antibody. Blots were quantitated using a Licor Odyssey scanner, and the amount of arrestin2 bound to GST-clathrin-(1−363) was normalized to the level of arrestin overexpression. Binding to various arrestin2 mutants was compared to full-length arrestin2 (set to 1). Representative Western blots showing binding and arrestin expression (input) are shown. Bars represent the mean ± SE from nine independent experiments. Statistical analysis was performed using a paired *t* test (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ versus full length).

Interestingly, despite the poor expression of arrestin2-(319−418) compared to full-length arrestin2 (compare protein levels in the input lanes), arrestin2-(319−418) showed a significant increase in binding to both GST-clathrin-(1−363) and GST-β_2-adaptin (Figure 1A). This increase was particularly evident with clathrin binding and suggests that the arrestin2 holoprotein contains determinants that inhibit clathrin binding and that this regulation is lost when only the C-tail is present. Thus, we wanted to study the role of the arrestin2 C-tail, in the context of full-length arrestin2, on clathrin binding. To do this, we initially analyzed the binding of purified full-length and C-terminally truncated (residues 1−393) arrestin2 to GST-clathrin-(1−363). Bovine arrestin-2 was expressed and purified from bacteria as previously described (*16*). Truncation of the C-terminal 25 amino acids in arrestin2 led to an ~3-fold increase in clathrin binding compared to full length, suggesting that the C-tail of arrestin2 plays an inhibitory role in clathrin binding (Figure 1B). In order to further analyze this potential inhibitory role, we constructed several truncations to progressively shorten the tail (Figure 1C). The truncated arrestins were expressed in Cos 1 cells and analyzed for binding to GST-clathrin-(1−363). Two regions within the C-terminal tail appeared to play a role in inhibiting clathrin binding, with arrestin2-(1−408) and arrestin2-(1−400) showing an ~4-fold and ~12-fold increase in binding to GST-clathrin-(1−363), respectively (Figure 1D). Interestingly, deletion of residues 401−408, which we term the acidic patch due to the large number of acidic residues, led to the largest increase in clathrin binding. Consistent with our binding data using purified proteins, expressed arrestin2-(1−393) had enhanced clathrin binding, being ~7-fold higher than full-length arrestin2. Interestingly, these C-terminal truncations had a lesser, albeit still significant, effect on binding to GST-β_2-adaptin, with arrestin2-(1−408) and arrestin2-(1−400) showing ~2-fold and ~3-fold increased binding (Figure 1E). Arrestin2-(1−393) did not bind to β_2-adaptin as previously reported (*10*).

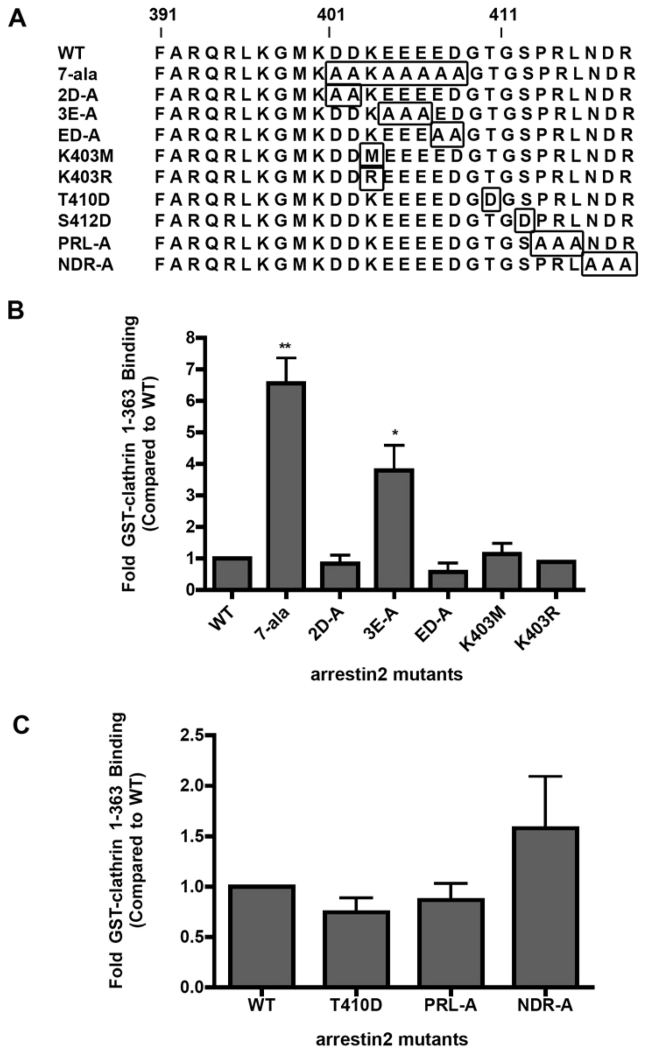

FIGURE 2: The acidic patch contains key residues involved in regulating clathrin binding. (A) Sequences of the arrestin2 C-tail mutations used in the binding assays. Residues characterized in this study are boxed. (B) Analysis of the role of the "acidic patch" in regulating clathrin binding. (C) Analysis of the role of the extreme C-tail in regulating clathrin binding. GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate overexpressing wild-type or mutant arrestin2 as described in Materials and Methods. Bars represent the mean ± SE from four to six independent experiments. Statistical analysis was performed using a paired t test (*, $p < 0.05$; **, $p < 0.005$ versus full length).

To analyze the role of individual residues within the C-tail, we performed site-directed mutagenesis on the region spanning residues 401−418 (Figure 2A). First, to analyze the contribution of the acidic patch, we generated an arrestin2 mutant in which all acidic residues between 401 and 408 were mutated to alanine (7-ala). This mutant showed an ~7-fold increase in clathrin binding, suggesting that these residues contribute to the inhibitory nature of the C-tail (Figure 2B). To further narrow down the residues involved, we performed alanine scanning of this region. Of all the combinations tested, mutation of the three glutamic acid residues at positions 404−406 to alanine (3E-A) had the largest effect on clathrin binding, showing an ~4-fold increase in binding compared to wild-type arrestin2. In contrast, mutation of Asp-401 and Asp-402 to alanine (2D-A) or Glu-407 and Asp-408 to alanine (ED-A) had no effect on clathrin binding (Figure 2B). We also analyzed the role of the lone basic residue located within the acidic patch, Lys-403. Arrestin2 has previously been shown to be ubiquitinated on multiple lysine residues, and it has been suggested that this modification is essential for its endocytic functions (*17*). Although we do not know whether Lys-403 of arrestin2 is ubiquitinated in Cos 1 cells, we wanted to determine if mutation of this residue would affect clathrin binding. We mutated Lys-403 to either arginine (arr2-K403R), to maintain the charge but prevent modifications such as ubiquitination, or methionine (arr2-K403M), to remove the charge but maintain the size of the side chain. Neither of these mutations had an effect on clathrin binding, suggesting that this lone basic residue within the acidic patch does not play a role in regulating clathrin binding (Figure 2B). Taken together, these results suggest that Glu-404, Glu-405, and Glu-406 are the key residues within the acidic patch involved in regulating clathrin binding.

We next tested the contribution of residues 409−418 within the C-tail. We generated a Thr-410 phospho mimic, T410D, and performed triple alanine scanning of the last six amino acids. None of these mutants significantly affected clathrin binding (Figure 2C). Since these mutations did not increase the levels of clathrin binding comparable to the 4-fold increase seen with deletion of this region, this suggests that a major effect of truncating residues 409−418 may involve the destabilization of other regions of arrestin2.

Effect of Arrestin2 Phosphorylation on Clathrin Binding. Previous studies have shown ERK1/2-mediated phosphorylation of Ser-412 in arrestin2 under basal conditions (*18*, *19*). In these studies, overexpression of an arrestin phospho mimic, S412D, failed to promote arrestin-mediated β_2AR internalization. In addition, coimmunoprecipitation from cell lysates revealed decreased binding of arrestin2-S412D to the clathrin heavy chain, suggesting that phosphorylation of Ser-412 regulates arrestins' endocytic function (*17*). No studies, however, have analyzed the direct binding of the phospho mimic to clathrin. To determine if the negative charge on Ser-412 directly affects clathrin binding, we analyzed the binding of purified wild-type and S412D mutant arrestin2 to GST-clathrin-(1−363). The use of purified arrestin2 allowed us to directly analyze binding to clathrin, removing other potential interacting proteins that may be present within the cell. Purified arrestin2-S412D showed a decreased ability to bind clathrin, with an ~80% reduction compared to wild type (Figure 3A). In contrast, purified arrestin2-S412D bound to GST-β_2-adaptin with levels similar to wild-type arrestin2 (Figure 3A).

We next tested two different Ser-412 mutants to further investigate the role of arrestin2 phosphorylation in clathrin binding. These mutants were overexpressed in Cos 1 cells and analyzed for their ability to bind GST-clathrin-(1−363) (Figure 3B). As with our purified proteins, arrestin2-S412D from a cell lysate had significantly reduced clathrin binding, with an ~80% reduction compared to wild type. Interestingly, arrestin2-S412A, which is unable to be phosphorylated at this position, also showed a large reduction in clathrin binding. These results suggest that the potential loss of hydrogen bonds upon mutation of Ser-412 to alanine and the formation of ionic interactions upon mutation to aspartate are important factors in reducing clathrin binding.

Effect of Arrestin2 Activation on the C-Terminal Tail Mediated Inhibition. Binding of arrestin to the phosphorylated activated receptor is thought to induce a conformational change, switching arrestin from an inactive basal state to an activated state (*20*, *21*). This activation is believed to result in extension of the C-tail and exposure of the clathrin and AP-2 binding sites (*10*).

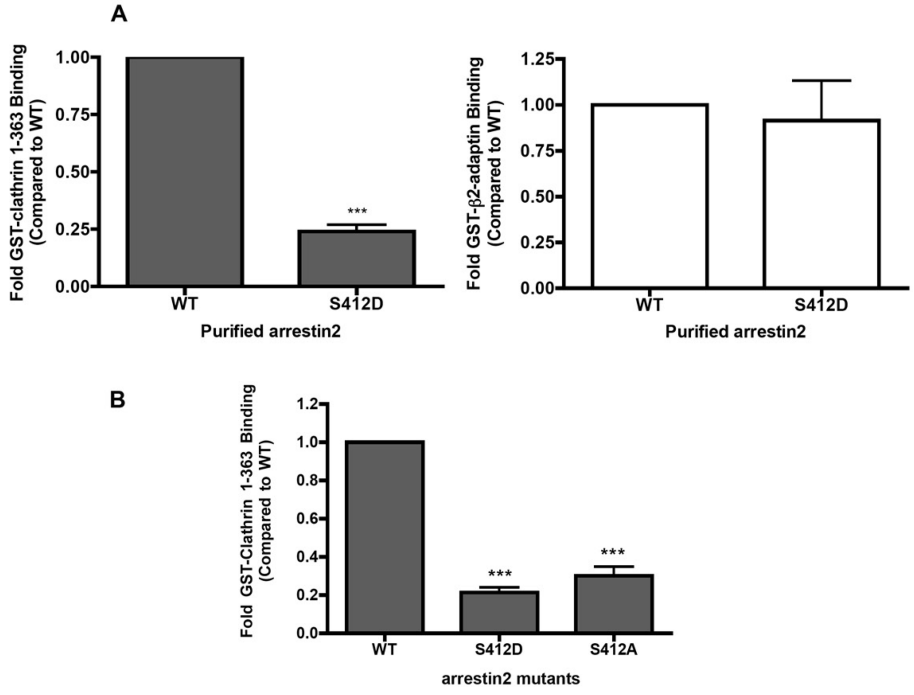

FIGURE 3: Analysis of the arrestin2-S412 mutants on clathrin binding. (A) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) (left panel) or GST-β_2-adaptin (700−937) (right panel) and 10 pmol of purified arrestin2. (B) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate overexpressing wild-type or mutant arrestin2 as described in Materials and Methods. Bars represent the mean ± SE from six to nine independent experiments. Statistical analysis was performed using a paired *t* test (**, $p <$ 0.005; ***, $p <$ 0.0005 versus full length).

Charge inversion of a key arginine residue within the polar core of arrestin2, R169E, generates a mutant that mimics this activated state (*22*). To determine if arrestin2 activation can overcome the inhibitory constraints within the C-terminal tail, the R169E mutation was incorporated into arrestin2-S412D and the various truncations (Figure 4). Arrestin2-R169E showed dramatically increased binding to GST-clathrin-(1−363), with an ~200-fold increase compared to wild-type arrestin2 (Figure 4A). Interestingly, combining the R169E and S412D mutations (R169E-S412D) also led to an ~200-fold increase in clathrin binding compared to wild type. Since we observed no significant difference in the level of clathrin binding between R169E and R169E/S412D, these results suggest that the R169E mutation can overcome the inhibitory nature of the S412D mutation. Incorporation of the R169E mutation into the C-tail truncations also led to a further increase in clathrin binding compared to the truncations alone (Figure 4B). These results suggest that activation of arrestin2 completely overcomes the inhibition mediated by the C-tail and that dephosphorylation of arrestin2 might not be required to promote clathrin binding.

The N-Terminus of Arrestin2 Contributes to Clathrin Regulation. To aid in detection of the various arrestin2 C-terminal mutations and truncations, we generated constructs containing an N-terminal hemagglutinin (HA) epitope tag. To our surprise, addition of the N-terminal HA tag led to an ~30-fold increase in both clathrin and β_2-adaptin binding compared to untagged arrestin2 (Figure 5A). Interestingly, truncation of the HA-arrestin2 C-tail, HA-arrestin2-(1−400), led to an ~60% reduction in binding to clathrin and β_2-adaptin compared to full-length HA-arrestin2 (Figure 5A). This is opposite to what we observed with the untagged truncations (compare Figures 1B and 5A). Binding of HA-arrestin2-(1−400), however, was still ~13-fold and ~10-fold higher for clathrin and β_2-adaptin, respectively, compared to untagged arrestin2 (Figure 5A). These results suggest that the N-terminus of arrestin2 regulates clathrin binding and that this regulation is disrupted by the addition of an HA tag. Reduced binding of the HA-tagged arrestin2 upon truncation of the C-tail raises the possibility that these two regions interact. Interestingly, despite the large increase in clathrin binding observed with HA-arrestin2, we observed a further significant increase upon addition of the R169E mutation into HA-arrestin2 (HA-R169E) (Figure 5B). These results suggest that arrestin activation, mimicked by the R169E mutation, is still the main driving force in promoting clathrin binding.

The amino acid sequences of arrestin2 and -3 are ~78% identical (*23*). Interestingly, addition of an HA tag onto the N-terminus of arrestin3 did not significantly affect binding to GST-clathrin-(1−363) (Figure 5C). Analysis of the N- and C-terminal regions of the two nonvisual arrestins reveals that while the basic residues within the N-terminus are conserved, the C-tails are very different (Figure 5D). These results suggest that the inhibitory mechanism involving the N-terminus is selectively present in arrestin2 and that this mechanism may involve the C-terminal domain of arrestin2.

Unlike the acidic C-tail, the N-terminus of arrestin2 is basic, with four of the first eleven residues (Lys-4, Arg-7, Lys-10, and Lys-11) being basic (Figure 5D). To analyze the role of the basic residues within the N-terminus, we performed site-directed mutagenesis and then analyzed clathrin binding (Figure 6A). First, to determine if the basic residues are involved in regulating clathrin binding, we generated an arrestin2 mutant in which all four basic residues were mutated to alanine. This mutant, termed 4A, showed an ~8-fold increase in clathrin binding compared to wild-type arrestin2 (Figure 6B). Mutation of these individual basic residues led to an ~6-fold, ~9-fold, ~43-fold, and ~4-fold increase in clathrin binding for K4A, R7A, K10A, and K11A,

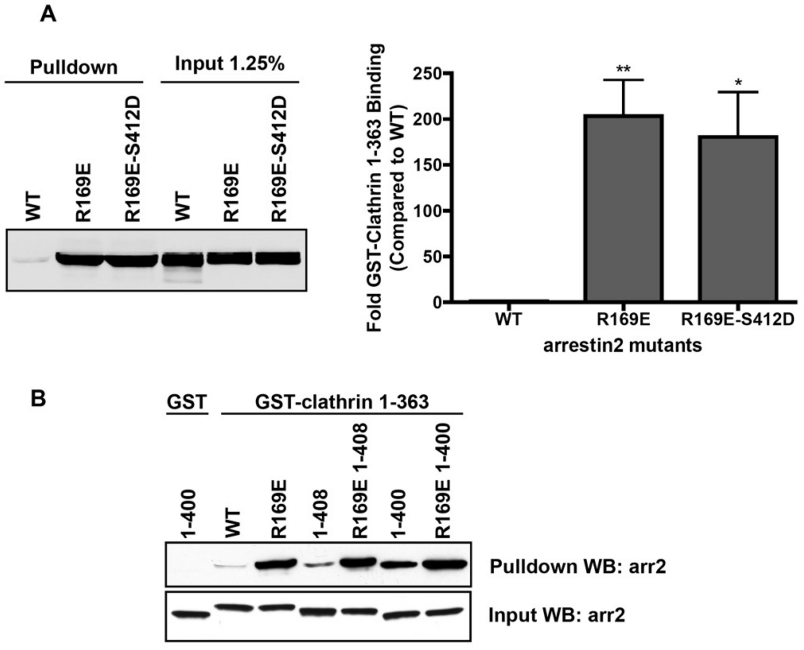

FIGURE 4: Arrestin2 "activation" relieves inhibitory constraints of the C-tail. (A) Effect of the R169E mutation on the binding of arrestin2-S412D to GST-clathrin-(1−363). GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate overexpressing wild-type or mutant arrestin2. Representative Western blot (left panel) and quantitation (right panel) are shown. Bars represent the mean ± SE from six to eight independent experiments. Statistical analysis was performed using a paired t test (*, $p < 0.05$; **, $p < 0.005$ versus full length). (B) Effect of the R169E mutation on the binding of C-terminally truncated arrestin2 to GST-clathrin-(1−363). GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate overexpressing wild-type or mutant arrestin2. Arrestin2 binding (upper panel) and input (lower panel) are shown.

respectively. These results suggest that these four basic residues play a major role in regulating clathrin binding, with Lys-10 having the largest effect. We next mutated Lys-10 to a glutamate (K10E) to determine if the positive charge at this position was mediating this effect. This mutant showed an ~7-fold increase in binding to GST-clathrin-(1−363), much lower than that seen for the K10A mutant, suggesting that hydrogen bonding as well as ionic interactions is important (Figure 6C). Interestingly, the K10A mutation also showed enhanced binding to GST-β_2-adaptin, although the increase was lower than that seen with clathrin binding (Figure 6C). Overall, these results suggest that the basic residues within the N-terminus of arrestin2 are involved in interactions that contribute to the basal regulation of clathrin binding, with Lys-10 playing a major role.

Analysis of the arrestin2 structure reveals that Lys-10 and Lys-11 are in close proximity to several residues located just N-terminal to the acidic patch (Figure 7A). The structure of this region enables us to determine distances between the side chains and predict the formation of hydrophobic (bold dotted lines) and hydrogen bonds (small dotted lines). This reveals that Lys-10 may form hydrophobic interactions with Phe-388 and hydrogen bonds with Asp-390 and Tyr-21 while Lys-11 may form hydrophobic interactions with Phe-391, Val-164, and Leu-166. In addition, Arg-7 may form hydrogen bonds with Glu-389 (not shown). This suggests that these residues likely participate in a series of intramolecular interactions in the basal state. Previous studies have shown that the individual E389R and D390R mutants have no effect on binding to GST-clathrin-(1−579) or GST-β_2-adaptin (*16*). The structure of arrestin2, however, suggests that a single mutation of Glu-389 or Asp-390 might not be sufficient to disrupt this region due to the numerous intramolecular interactions. To analyze the contribution of these residues, we generated a mutant in which the charge at both Glu-389 and Asp-390 was reversed (E389R/D390R). This mutation should affect clathrin binding if these residues are involved in stabilizing the N-terminus or C-tail. Indeed, the arrestin2-E389R/D390R double mutant showed an ~4-fold increase in clathrin binding (Figure 7B). These results suggest that Glu-389 and Asp-390 play a role in inhibiting clathrin binding likely through the formation of a series of hydrophobic and hydrogen bonds with the N-terminal basic residues. In this model, we propose the formation of a series of intramolecular interactions between the N- and C-terminal regions, which are further stabilized through interactions with numerous surrounding residues. This would keep the N-terminus and C-tail locked in position until these interactions are disrupted.

Effect of N- and C-Terminal Arrestin2 Mutants on Receptor Internalization. We next wanted to test whether the arrestin2 mutants had an effect on the internalization of GPCRs. To test this, Cos 1 cells were transiently transfected with FLAG-β_2AR and various arrestin2 constructs and treated with isoproterenol, and ELISA assays were performed to measure the loss of cell surface receptors. Wild-type and mutant arrestins were expressed at comparable levels in these cells (not shown). Overexpression of wild-type arrestin2 led to an ~2−3-fold increase in Flag-β_2AR internalization at 5, 10, and 20 min compared to mock-transfected cells (Figure 8). The various arrestin2 mutants were comparable to wild-type arrestin2 in their ability to promote β_2AR internalization at all time points tested. Thus, the increased ability of these mutants to bind clathrin *in vitro* did not affect the overall ability of these mutants to promote β_2AR internalization.

DISCUSSION

Previous studies have suggested an interaction between the basic N- and acidic C-terminal regions of arrestin (*24*, *25*), although this has not been directly shown and much of these

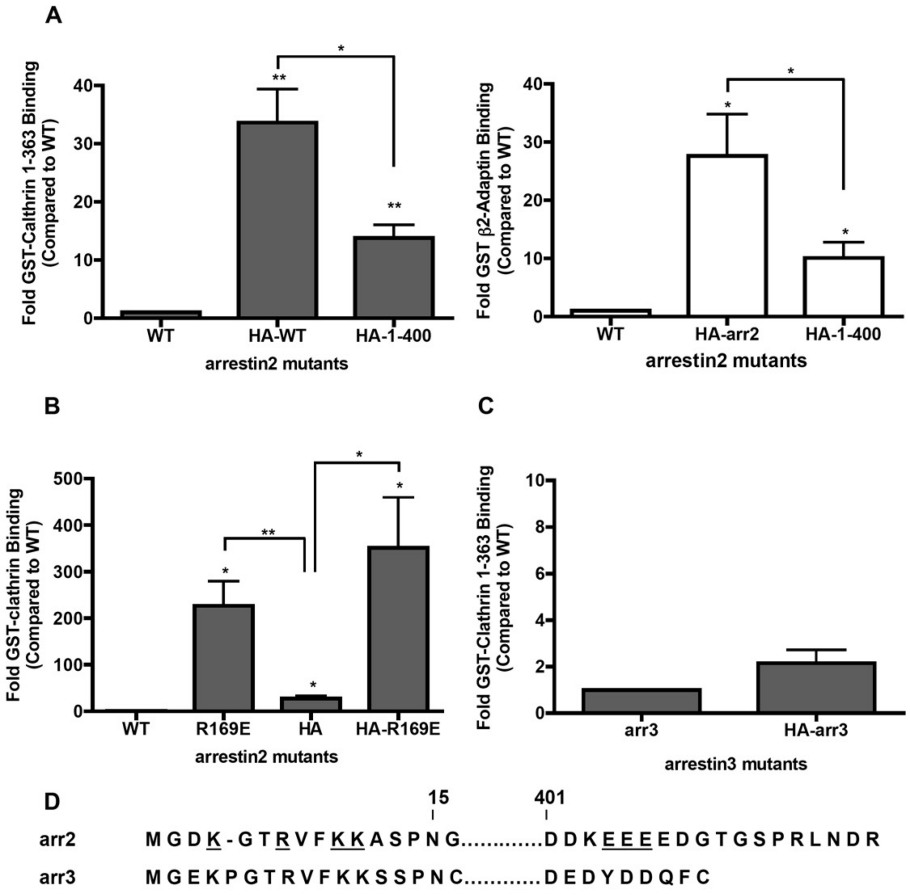

FIGURE 5: Addition of an N-terminal HA tag increases binding of arrestin2 to clathrin. (A) Effect of an N-terminal HA tag on binding of full-length arrestin2 or arrestin2-(1−400) to GST-clathrin-(1−363) (left panel) or GST-β_2-adaptin-(700−937) (right panel). The bars represent the mean ± SE from three independent experiments. (B) Effect of the R169E mutation on HA-arrestin2 binding to GST-clathrin-(1−363). Bars represent the mean ± SE from five independent experiments. (C) Comparison of untagged vs HA-tagged arrestin3 binding to GST-clathrin-(1−363). Bars represent the mean ± SE from ten independent experiments. (D) Alignment of the arrestin2 and arrestin3 N- and C-terminal regions. Statistical analysis was performed using a paired *t* test (*, $p < 0.05$; **, $p < 0.005$ versus full length).

regions are either unstructured (*16*) or absent (*26*) in the published arrestin2 crystal structures. Here, we provide evidence that the N-terminus and C-terminal tail of arrestin2 are involved in regulating the ability of arrestin2 to interact with clathrin, likely through the formation of intramolecular interactions. The arrestin2 C-tail was found to play an inhibitory role in clathrin binding with two regions mediating this inhibition, residues 401−408 and 409−418. Mutagenesis revealed that three acidic residues within the tail, Glu-404, Glu-405, and Glu-406, are key residues that inhibit clathrin binding while mutation of Ser-412 to Asp (to mimic phosphorylation) also led to decreased clathrin binding. We also identified an inhibitory role for several basic residues within the N-terminus, Lys-4, Arg-7, Lys-10, and Lys-11, in clathrin binding. Comparison of arrestin2 and arrestin3 binding to clathrin suggests that this mode of regulation is specific for arrestin2. Overall, these results shed light on how the basal interaction between arrestin2 and clathrin is regulated.

In the basal state, Ser-412 of arrestin2 is phosphorylated by ERK1/2 (*19*). Previous studies have suggested that this phosphorylation functions to inhibit clathrin binding and that dephosphorylation is required to promote clathrin binding and internalization (*18*, *19*). However, our results suggest that activation of arrestin2 may be the main driving force that promotes clathrin binding and that dephosphorylation may not be required at this step. First, we found that an arrestin2 mutant that is unable to be phosphorylated, arr2-S412A, showed decreased clathrin binding compared to wild type, similar to what we observed with the phospho mimic arrestin2-S142D. These results suggest that hydrogen and ionic interactions at Ser-412 are important in regulating clathrin binding. We propose that the C-tail of arrestin2 is involved in intramolecular interactions that inhibit clathrin binding and that phosphorylation of Ser-412 stabilizes these interactions. Second, we show that while arrestin2-S412D shows decreased clathrin binding, the arrestin2-R169E-S412D double mutant, which mimics the "active" phosphorylated form of arrestin2, overcomes this defect and shows enhanced clathrin binding similar to arrestin2-R169E. Incorporation of the R169E mutant into the various arrestin2 C-tail truncations also resulted in increased clathrin binding compared to the truncations alone. These results suggest that activation of arrestin2 overcomes the inhibitory constraints contained within the arrestin2 C-tail. These results, however, do not rule out the possibility that arrestin2 dephosphorylation is necessary to promote some other step in the internalization process, since the S412D mutant has been shown to be defective in promoting internalization (*18*, *19*).

Previous studies have reported increased clathrin binding with the arrestin2-S412A mutant (*18*). There are several possible explanations as to why our results are conflicting with these studies. Binding of arrestin2-S412A to clathrin was previously performed using coimmunoprecipitations (*18*), whereas we analyzed binding using GST-pulldown assays. In our system, we

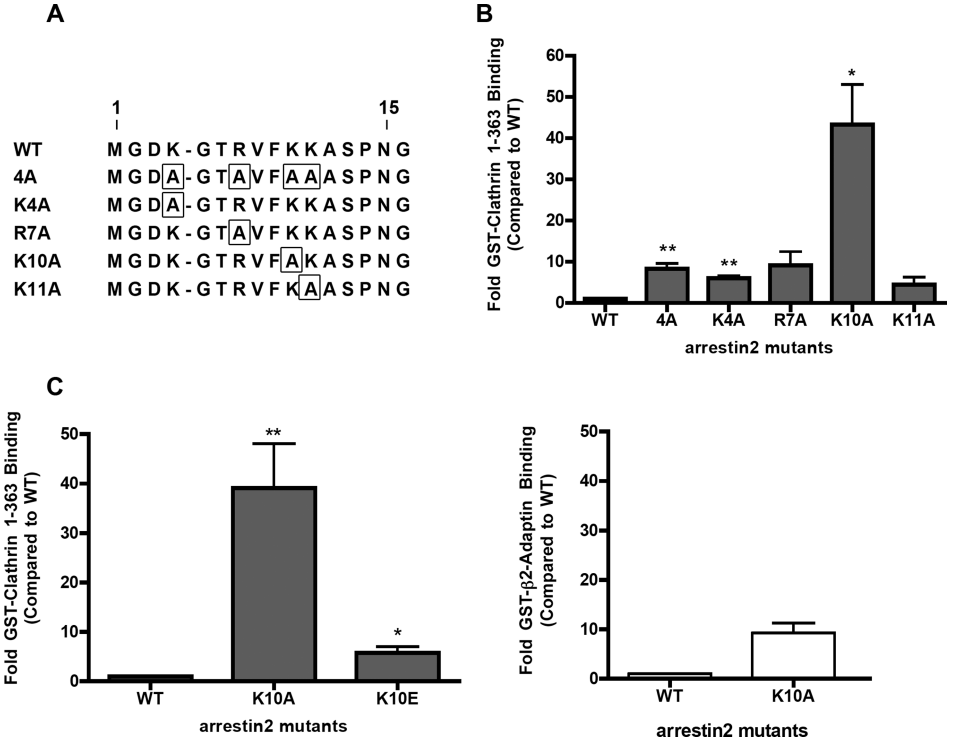

FIGURE 6: The arrestin2 N-terminus regulates clathrin binding. (A) Sequences of the arrestin2 N-terminal mutations used in the binding assays. Residues characterized in this study are boxed. (B) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate containing wild-type or mutant arrestin2. (C) Analysis of the arrestin2 Lys-10 mutant binding to GST-clathrin-(1−363) (left panel) or GST-β_2-adaptin-(700−937) (right panel). Bars represent the mean ± SE from four to six independent experiments. Statistical analysis was performed using a paired t test (*, $p < 0.05$; **, $p < 0.005$ versus full length).

selectively used the β-propeller region of the clathrin terminal domain, GST-clathrin-(1−363), which contains the main arrestin2 binding site (*11*). Using this method we were able to use purified clathrin, removing other cellular components that interact with clathrin and arrestin2, such as AP-2. It is therefore possible that the previous results show increased arrestin2 binding to clathrin due to the interaction with other proteins. Also, use of C-terminally FLAG-tagged arrestin2 or overexpression of a dominant negative MEK (K97A) was shown to enhance the arrestin2-Flag/clathrin interaction (*19*). Although it was not tested, it is possible that a C-terminal tag on arrestin2 would have a similar effect as observed with the N-terminal tag, leading to an increase in clathrin binding.

Our results also suggest a role for the N-terminus of arrestin2 in preventing the basal interaction with clathrin. Addition of a HA tag onto the N-terminus of arrestin2 led to a dramatic increase in clathrin binding, possibly through the destabilization of intramolecular interactions involving the N-terminus. Site-directed mutagenesis revealed that four basic residues, Lys-4, Arg-7, Lys-10, and Lys-11, are involved in this regulation. Previous studies have shown that Lys-14 and Lys-15 within the N-terminus of arrestin1 serve as the initial phospho sensors and are involved in the discrimination of the phosphorylation status of rhodopsin (*13*). These studies suggested that these two residues recognize the phosphorylated form of the receptor and guide the phosphate into the polar core. Since Lys-14 and Lys-15 of arrestin1 correspond to Lys-10 and Lys-11 of arrestin2, it is likely that Lys-10 and Lys-11 serve as phospho sensors in arrestin2. Taken together, these results suggest that disruption of the arrestin2 phospho sensor is a key step in initiating clathrin binding.

Interestingly, Lys-10 and Lys-11 in arrestin2 correspond to Lys-11 and Lys-12 in arrestin3, which have been shown to be ubiquitinated following GPCR activation (*27*). The ubiquitination of Lys-11 and Lys-12 has been shown to regulate the endocytic and scaffolding functions of arrestin3 following stimulation of the $AT_{1A}R$ (*27*). Our results show that mutation of Lys-10 and Lys-11 enhances the binding of arrestin2 to clathrin and AP-2. While we currently do not know the role, if any, of N-terminal arrestin2 ubiquitination, it is possible that the ubiquitination of the N-terminal arrestin3 lysines mediates a similar effect as our arrestin2 N-terminal mutations or HA-tag, namely, helping to maintain arrestin in an open conformation to allow enhanced binding to downstream components.

It is interesting that addition of an N-terminal HA tag onto arrestin3 had no significant effect on clathrin binding. This suggests that regulation of the interaction between clathrin and arrestin3 might differ from that of arrestin2. This is interesting in light of the fact that the nonvisual arrestins have been shown to exhibit different dynamics in their ability to bind clathrin and promote GPCR internalization. For example, arrestin3 is ~100-fold more effective at promoting β_2AR internalization in mouse embryonic fibroblasts compared to arrestin2 (*28*), even though arrestin2 and -3 bind to the β_2AR with comparable affinity (*29*). This suggests that the difference in internalization might be due to differences in their binding to endocytic proteins. In fact, analysis of nonvisual arrestin binding to purified clathrin cages revealed that arrestin3 binds with higher affinity with a K_d of 10 nM versus 64 nM for arrestin2 (*7*). In addition, intact cell studies showed enhanced GPCR-promoted interaction between arrestin3 and β2-adaptin compared with arrestin2/β2-adaptin, suggesting that a lower level of arrestin2 is recruited into clathrin-coated pits following receptor stimulation (*30*). Sequence analysis reveals that while the N-terminal basic residues are fully conserved between the nonvisual arrestins, there are several major

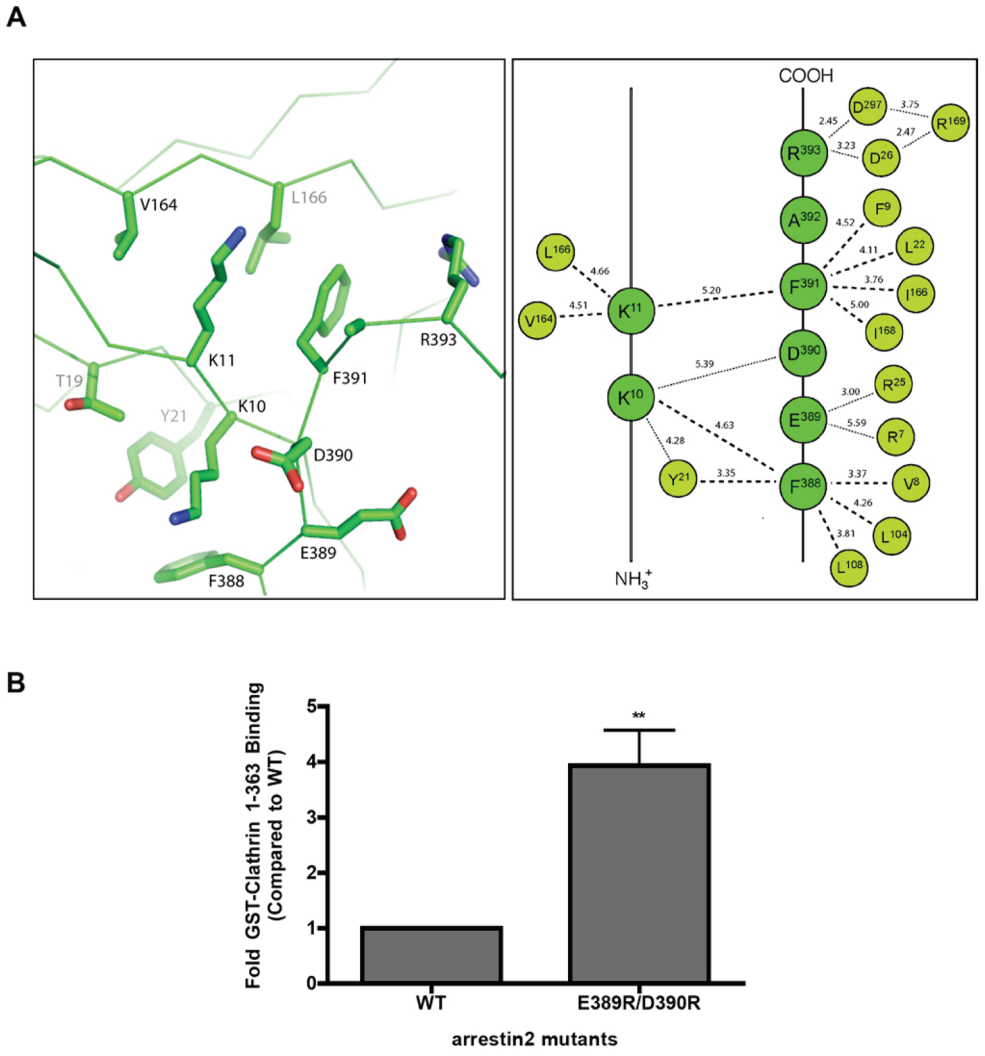

FIGURE 7: Modeling and binding analysis of Lys-10 and Lys-11 interactions. (A) Structure of arrestin2 highlighting residues surrounding Lys-10 and Lys-11 (left panel). Color of residues is as follows: nitrogen = blue and oxygen = red. Model of predicted intramolecular interactions formed between the N-terminus and the C-terminal tail (right panel). Bold dotted lines represent potential hydrophobic interactions, while smaller dotted lines represent potential hydrogen bonds. The distances between residues are noted in angstroms. (B) GST pulldowns were performed using 100 pmol of GST-clathrin-(1−363) and 100 μg of Cos 1 cell lysate containing wild-type or mutant arrestin2. Bars represent the mean ± SE from five independent experiments. Statistical analysis was performed using a paired *t* test (**, $p < 0.005$ versus full length).

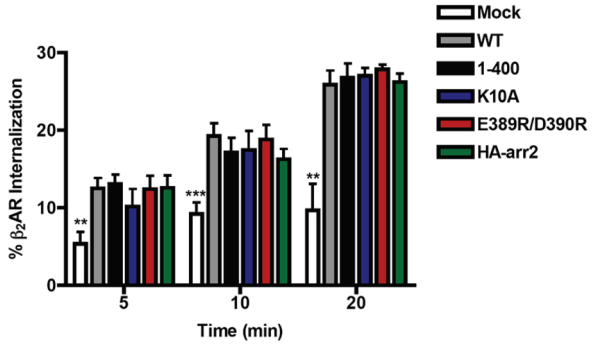

FIGURE 8: Internalization of β_2AR in Cos 1 cells overexpressing wild-type and mutant arrestin2. Cos 1 cells were transiently transfected with Flag-β_2AR and wild-type or mutant arrestin2. Forty-eight hours posttransfection the cells were treated with or without 10 μM isoproterenol for 5, 10, or 20 min at 37 °C. β_2AR internalization was measured by ELISA as described under Materials and Methods. Bars represent the mean ± SE from six to ten independent experiments. Statistical analysis was performed using a paired *t* test (**, $p < 0.005$; ***, $p < 0.0005$ versus wild type).

differences between the C-tails: (1) arrestin3 is nine amino acids shorter and is overall less acidic; (2) the residue at position 404, which is one of the three residues within the acidic patch of arrestin2 that regulates clathrin binding, is replaced by a tyrosine in arrestin3; and (3) the ERK1/2 phosphorylation site, Ser-412, is absent in arrestin3. It is intriguing to speculate that the basal conformations of arrestin2 and -3 are different, with arrestin3 being more "available" to bind clathrin. While the question still remains as to why arrestin2 has more inhibitory constraints to overcome compared to arrestin3, this may reflect the downstream activities of arrestins following internalization, allowing differential temporal and functional activities to be achieved.

Interestingly, recent studies have also suggested that arrestin2 and -3 undergo different conformational changes following activation (*31*, *32*). Using a phosphorylated peptide derived from the V_2 vasopressin receptor, it was shown that the N-terminal domain of arrestin2 is protected from trypsin-mediated cleavage following activation, while both the N- and C-terminal domains of arrestin3 are protected (*32*). Also, Arg-8 in arrestin3 is cleaved by trypsin; however, following peptide binding this residue

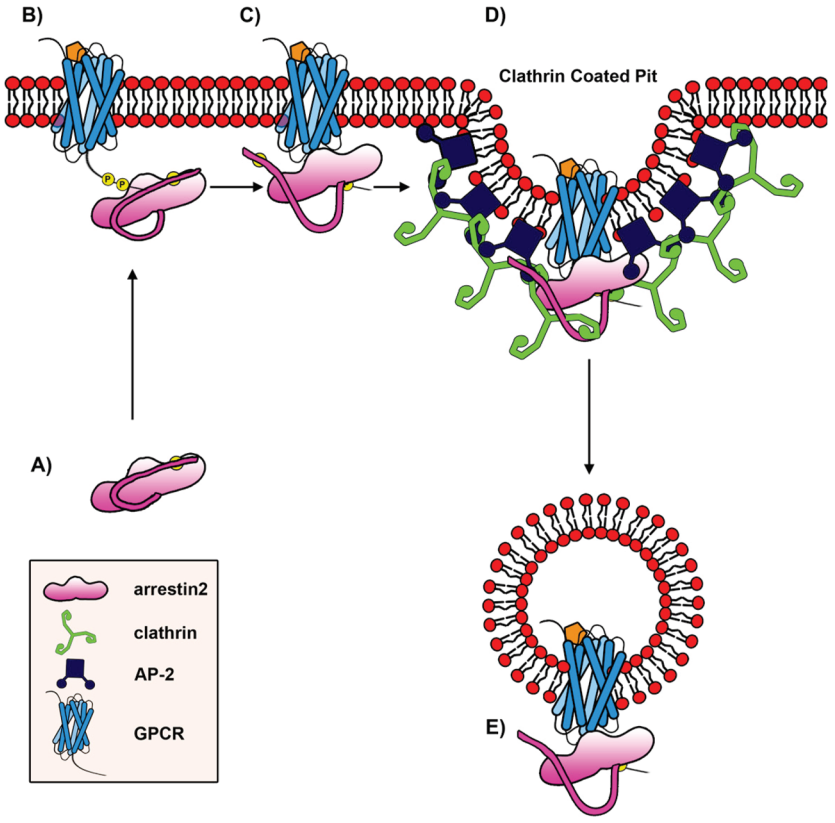

FIGURE 9: Model of the regulation of the basal arrestin2/clathrin interaction. (A) In the basal state, the N-terminus and C-tail form various intramolecular interactions within arrestin2. The phosphate group at Ser-412 helps to stabilize these interactions. In this state, the loop that contains the clathrin-binding box is in a "closed" conformation and is inaccessible to clathrin binding. (B) Upon receptor binding, the phosphate groups on the receptor bind to Lys-10 and Lys-11, disrupting the intramolecular interactions involving the N-terminus, leading to an "intermediate" state that exhibits increased clathrin binding. In this state, however, the C-tail is still inaccessible and clathrin binding is not maintained. (C) The phosphate groups are guided into the polar core, resulting in a conformational change that disrupts the intramolecular interactions involving the C-tail. The C-tail becomes fully extended, "opening" the loop and the clathrin-binding site. Arrestin is now able to recruit the GPCR to clathrin-coated pits via clathrin and AP-2 binding. (D) In the clathrin-coated pit, the clathrin-binding box engages the clathrin terminal domain, while the C-tail of arrestin binds to β2-adaptin. (E) The receptor is internalized.

becomes inaccessible to trypsin cleavage, suggesting that the N-terminus undergoes a conformation change in which the Arg becomes buried (*32*). Surprisingly, Arg-7 of arrestin2, which we identified as playing a role in regulating clathrin binding, was not shown to be accessible to cleavage before or after peptide-mediated activation (*32*). This suggests that the N-terminal domains of arrestin2 and -3 are in different conformations, possibly explaining why addition of a HA tag only affected arrestin2 binding to clathrin. If the basal states of arrestin2 and -3 are identical, one would predict that they would undergo similar conformation changes following receptor binding, due to sequence similarities and conservation of critical functional domains. The data presented here, along with the previous studies comparing the activation-dependent conformational changes, lead us to predict that arrestin2 and -3 have different basal conformations, most likely resulting from a different network of intramolecular interactions.

Several of the N- and C-terminal arrestin2 mutants were analyzed for the ability to promote β_2AR internalization. Surprisingly, none of the mutants tested appeared to differ from wild-type arrestin2 in being able to promote β_2AR internalization. There are a few possibilities to explain this discrepancy. One likely possibility is that while the arrestin2 mutants have an enhanced ability to bind to clathrin *in vitro*, these mutants obviously still need to bind to the receptor to promote endocytosis in cells. Since receptor binding is thought to promote arrestin activation, this binding would overcome the advantage that an enhanced clathrin binding mutant would provide. This is supported by our findings that addition of the R169E mutation onto several of our mutants led to dramatic increases in clathrin binding. Another possibility relates to the finding that GPCRs can be divided into two classes based on their arrestin binding properties (*33*, *34*). Arrestins bind to "class A" receptors, such as the β_2AR, with relatively low affinity and dissociate soon after the receptor internalizes. On the other hand, arrestins bind to "class B" receptors, such as the angiotensin II type 1a receptor ($AT_{1A}R$), with higher affinity and remain associated with the receptor at the endosomes for prolonged periods of time (*27*, *33*, *34*). It is possible that our mutations do not affect the internalization of class A receptors but would instead alter the internalization of class B receptors. In fact, an arrestin3 K11R/K12R mutant, which shows reduced activation-dependent ubiquitination, had no effect on β_2AR trafficking but had a significant effect on $AT_{1A}R$ trafficking (*27*).

From these results we suggest the following model. In the basal inactive state, the C-tail of arrestin2 folds back against the main body with Glu-404, Glu-405, Glu-406, and phospho-Ser-412 forming intramolecular interactions with unknown regions (Figure 9). In addition, the N-terminal region of arrestin2 is involved in an intramolecular network of interactions with Lys-4, Arg-7, Lys-10, and Lys-11 playing a critical role. In this state, the loop containing the clathrin binding box (residues 376−380) is in

a conformation that does not support clathrin binding. Upon receptor activation and phosphorylation, arrestin2 binds to the receptor with Lys-10 and Lys-11 of arrestin2 interacting with the phosphate groups on the receptor. This leads to the disruption of the intramolecular interactions involving the N-terminus, resulting in an intermediate state that is partially accessible to clathrin binding. The phosphate is then guided into the polar core, leading to disruption of the polar core and the intramolecular interactions involving the C-tail, resulting in the extension of the tail and release of the clathrin-binding box and AP-2 binding regions.

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