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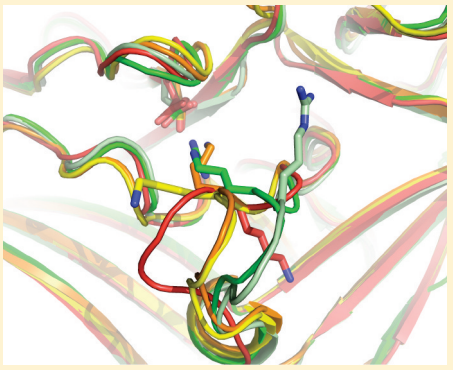
A Single Mutation in Arrestin-2 Prevents ERK1/2 Activation by Reducing c-Raf1 Binding

Sergio Coffa, Maya Breitman, Benjamin W. Spiller, and Vsevolod V. Gurevich*

Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232, United States

Supporting Information

ABSTRACT: Arrestins regulate the signaling and trafficking of G protein-coupled receptors (GPCRs). GPCR complexes with both nonvisual arrestins channel signaling to G protein-independent pathways, one of which is the activation of extracellular signal regulated kinase 1/2 (ERK1/2). Here we used alanine-scanning mutagenesis of residues on the nonreceptor-binding surface conserved between arrestin-2 and arrestin-3. We show that an Arg307Ala mutation significantly reduced arrestin-2 binding to c-Raf1, whereas the binding of the mutant to active phosphorylated receptor and downstream kinases MEK1 and ERK2 was not affected. In contrast to wild-type arrestin-2, the Arg307Ala mutant failed to rescue arrestin-dependent ERK1/2 activation via β2-adrenergic receptor in arrestin-2/3 double knockout mouse embryonic fibroblasts. Thus, Arg307 plays a specific role in arrestin-2 binding to c-Raf1 and is indispensable in the productive scaffolding of c-Raf1−MEK1−ERK1/2 signaling cascade. Arg307Ala mutation specifically eliminates arrestin-2 signaling through ERK, which makes arrestin-2−Arg307Ala the first signaling-biased arrestin mutant constructed. In the crystal structure the side chain of homologous arrestin-3 residue Lys308 points in a different direction. Alanine substitution of Lys308 does not significantly affect c-Raf1 binding to arrestin-3 and its ability to promote ERK1/2 activation, suggesting that the two nonvisual arrestins perform the same function via distinct molecular mechanisms.



Arrestins specifically bind active G protein-coupled receptors (GPCRs) phosphorylated by G protein-coupled receptor kinases and block further G protein activation (reviewed in refs 1 and 2). Bound arrestins link the receptor to the internalization machinery via interactions with clathrin[3] and AP2.[4] Arrestins also organize multiprotein signaling complexes, switching GPCR signaling to G protein-independent pathways (reviewed in refs 5 and 6). Arrestins scaffold mitogen activated protein kinases (MAPKs), facilitating the activation of JNK3,[7] ERK1/2,[8] and p38.[9] Free and receptor-bound arrestins differentially interact with MAPKs and other signaling proteins.[7−11] Here we focused on the c-Raf1−MEK1−ERK1/2 cascade, in which all three kinases bind arrestin-2 and arrestin-3[a].[12] ERK1/2 phosphorylation is facilitated by both nonvisual arrestins and is contingent on GPCR activation.[8] These data suggest that (a) the elements conserved between arrestin-2 and -3 play key roles and (b) the well-defined surface occupied by bound receptor[13−19] is not involved in the interactions with ERK or upstream kinases. Therefore, we performed alanine-scanning mutagenesis of the conserved residues on the nonreceptor-binding surface of arrestin-2 and compared the ability of wild-type (WT) and mutant arrestin-2 to bind receptor, c-Raf1, MEK1, ERK2, and promote ERK1/2 phosphorylation in cells. Our data identify Arg307 in arrestin-2 as a critically important component of c-Raf1 binding and ERK1/2 activation and show that the homologous Lys308 in arrestin-3 is not essential for these functions, suggesting that the two nonvisual arrestins scaffold Raf1−MEK1−ERK1/2 cascade via distinct molecular mechanisms.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP, [^{14}C]leucine, and [^{3}H]leucine were from Perkin-Elmer. All restriction enzymes were from New England Biolabs. Sepharose 2B and all other chemicals were from sources previously described.[20,21] Rabbit reticulocyte lysate was from Ambion, and SP6 RNA polymerase was prepared as described.[22] Rhodopsin was phosphorylated and regenerated by 11-*cis*-retinal generously supplied by Dr. R. K. Crouch (Medical University of South Carolina, Charleston, SC), as described.[23]

Arrestin Mutagenesis. Site-directed mutagenesis by PCR (Supporting Information Table S1) was performed using pGEM2-based transcription vectors encoding WT bovine arrestin-2 and arrestin-3 with engineered unique restriction sites described previously.[13,18] All constructs were verified by dideoxy sequencing. The coding sequences were excised with EcoR I and Hind III and subcloned into pcDNA3 for expression in cultured mammalian cells and into pFB vector for retrovirus production.

***In Vitro* Transcription, Translation, and Evaluation of Protein Stability.** Plasmids were linearized using a unique Hind III site downstream of the coding sequence. *In vitro* transcription and translation were performed as described.[24,25] All arrestin proteins were labeled by incorporation of

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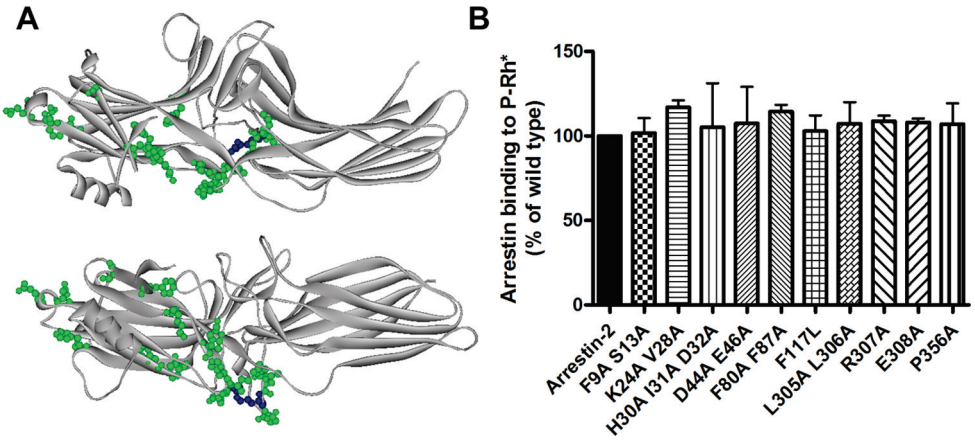

Figure 1. Ten arrestin-2 mutants retain normal receptor binding. (A) Arrestin-2 structure[44] viewed from the side (upper image) or convex surface (lower image). Residues on the nonreceptor-binding side, conserved in arrestin-2 and -3 that can be mutated to alanines without affecting receptor binding are shown as green CPK models, and Arg307 is shown in blue. (B) The binding of WT and mutant arrestin-2 to P-Rh*. Means ± SD of three experiments performed in duplicate are shown. ANOVA with Bonferroni post hoc test revealed no statistically significant differences between WT arrestin-2 and these mutants.

[^{3}H]leucine and [^{14}C]leucine with a specific activity of the mix of 1.5−3 Ci/mmol, resulting in the specific activity of arrestin proteins within the range of 66−85 Ci/mmol (150−230 dpm/fmol). The translation of every mutant used in this study produced a single-labeled protein band with the expected mobility on SDS-PAGE. Two parameters were used for the assessment of mutant relative stability, as described:[26] its yield multiplied by the percentage of the protein remaining in the supernatant after incubation for 10 min at 37 °C followed by centrifugation (Supporting Information Table S2).

Receptor Binding Assay. The binding to light-activated phosphorylated rhodopsin (P-Rh*) was performed, as described.[27] Briefly, translated radiolabeled arrestins (50 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol, 1 mM EGTA, 50 mM potassium acetate with 7.5 pmol (0.3 μg) of P-Rh* in a final volume of 50 μL for 5 min at 37 °C in room light and then cooled on ice. Bound and free arrestins were separated by size-exclusion chromatography on 2 mL columns of Sepharose 2B-CL equilibrated with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, at 4 °C. Rhodopsin-bound arrestins (eluted with receptor-containing membranes in the void volume between 0.5 and 1.1 mL) were quantified by liquid scintillation counting.

Co-immunoprecipitation and Western Blotting. Monkey kidney COS-7 cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen; Carlsbad, CA), according to the manufacturers protocol (3 μL of Lipofectamine 2000 per 1 μg of DNA). 24 h post-transfection, cells were serum-starved and lysed with lysis buffer (50 mM Tris, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM NaVO3, 10 mM *N*-ethylmaleimide, benzamidine, and phenylmethylsulfonylfluoride) on ice for 20 min. Cell debris were pelleted by centrifugation for 10 min at 10000*g*. Lysates were precleared with 30 μL of protein G agarose, followed by incubation with rabbit anti-FLAG antibody for 2 h and by the addition of 30 μL of protein G agarose beads for 2 h. The beads were then washed three times with lysis buffer, and bound proteins were eluted with Laemmli SDS buffer. In experiments involving ERK2, prior to lysis the cells were treated with 1 mM cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Pierce) for 30 min followed by 2 mM Tris-HCl, pH 7.5, for 15 min at room temperature. The proteins were separated by SDS PAGE (10%) and transferred to poly(vinylidene difluoride) membrane (Millipore, Bedford, MA). Blots were incubated with primary antibodies from Cell Signaling (mouse anti-HA (6E2) mAb #2367, 1:1500; mouse anti-p44/42 ERK1/2 (L34F12) mAb #4696, 1:1000; and mouse anti-p44/42 phospho-ERK1/2 (T202/Y204), (E10) mAb #9106S, 1:1000), or Sigma (mouse anti-FLAG M2, #F3165, 1:1500; rabbit anti-FLAG #F7425), followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch. Protein bands were visualized by enhanced chemiluminescence (ECL, Pierce) followed by exposure to X-ray film. The bands were quantified using VersaDoc with QuantityOne software (Bio-Rad Laboratories).

Arrestin-Dependent ERK Activation. For retrovirus production, human embryonic kidney (HEK) 293T cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA), according to the manufacturer's protocol (3 μL of Lipofectamine 2000 per 1 μg of DNA) with the following constructs: pVPack-GP (Stratagene, 217566), pVack-VSV-G (Stratagene, 217567), together with pFB-arrestin-2, pFB-arrestin-2-Arg307Ala, pFB-arrestin-3, pFB arrestin-3-K308A, or pFB-GFP. 24−48 h post-transfection, media containing the virus produced by HEK293T cells was collected and used to infect arrestin-2/3 double knockout mouse embryonic fibroblasts (MEFs) (a generous gift of Dr. R. J. Lefkowitz, Duke University).[28] Fresh virus-containing media was used daily for 3 days. Then MEFs were serum starved for 2 h and treated with 1 μM ICI118551, a biased ligand of β2-adrenergic receptor (β2AR), which is an inverse agonist of G protein signaling and an agonist of arrestin recruitment,[29] or 10 μM β2AR agonist isoproterenol for indicated time at 37 °C. MEFs were harvested and lysed in 50 mM Tris, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, supplemented with protease (Roche, 04693124001), and phosphatase (Roche, 04906845001) inhibitors cocktails on ice for 20 min.

RESULTS

To identify arrestin-2 elements involved in the binding of c-Raf1, MEK1, and ERK2, we generated 22 mutants in which residues conserved between nonvisual arrestins on the

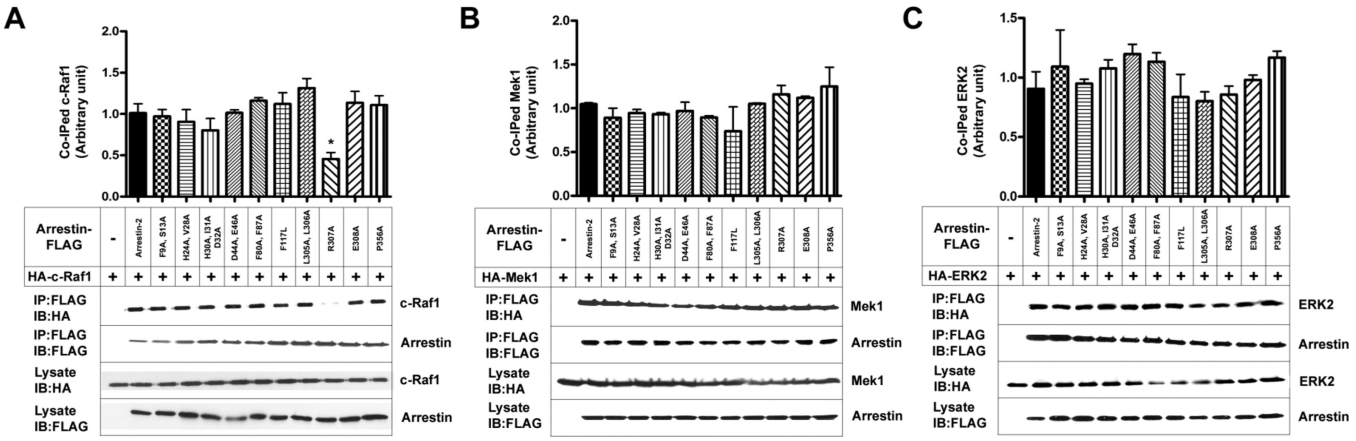

Figure 2. Binding of c-Raf1, MEK1, and ERK2 to WT and mutant arrestin-2. Flag-tagged WT arrestin-2 and indicated mutants were coexpressed with HA-tagged c-Raf1, MEK1, or ERK2 in COS7 cells. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immounoprecipitated c-Raf1 (A), MEK1 (B), or ERK2 (C) were visualized by Western blot with anti-HA antibody. The binding of all mutants to MEK1 and ERK2 was not different from WT arrestin-2, whereas Arg307Ala mutation significantly decreased the binding to c-Raf1. Means ± SD of 3−4 independent experiments are shown in bar graphs; representative blots are shown below. $^*p < 0.05$.

nonreceptor-binding surface were replaced with alanines individually or in groups (Figure 1A, Supporting Information Figure S1 and Table S1). Receptor binding is the signature function of arrestin proteins, which can be easily tested in a direct binding assay.[25,27] Both nonvisual arrestins demonstrate specific binding to phosphorylated light-activated rhodopsin (P-Rh*).[27,30,31] Therefore, we used binding to P-Rh* as the criterion to select proteins that interacted with the receptor normally. To this end, the mutants were expressed in cell-free translation, and their binding to P-Rh* was compared to that of WT arrestin-2 (Figure 1B, Supporting Information Figure S1). Twelve mutants showed significantly reduced binding, whereas ten demonstrated normal binding to P-Rh* (Supporting Information Table S2). These proteins were selected for subsequent experiments.

To compare the binding of WT and mutant arrestin-2 to c-Raf1, MEK1, and ERK2 in the cellular context, we transiently coexpressed Flag-tagged arrestins with HA-tagged kinases in COS-7 cells (chosen because they express very low levels of endogenous arrestins). Arrestins were immunoprecipitated with anti-Flag antibody, and co-immunoprecipitated kinases were detected by Western blotting using anti-HA antibody. We found that nine out of ten mutants bind c-Raf1 essentially as well as WT arrestin-2, whereas the amount of c-Raf1 co-immunoprecipitated with arrestin-2-Arg307Ala was significantly decreased (Figure 2A). Interestingly, none of the mutations tested affected arrestin-2 binding to MEK1 (Figure 2B) or ERK2 (Figure 2C). Thus, the Arg307Ala mutation selectively reduces arrestin-2 interaction with c-Raf1, without affecting the binding to the receptor or downstream kinases. Similar to other MAP kinases, c-Raf1 interacts with both domains of arrestin-2 and arrestin-3,[12] which suggests that its binding site includes more than one residue. However, the replacement of many residues on the putative kinase-binding surface with alanines reduces receptor binding (Supporting Information Figure S1). Therefore, their roles in kinase binding and activation could not be assessed by alanine scanning mutagenesis. Thus, while these data implicate Arg307 in c-Raf1 binding, they by no means suggest that it is the only arrestin-2 residue involved. Interestingly, an equivalent residue in arrestin-3, which also promotes ERK1/2 activation,[8] is Lys308 (Figure 3),[19]

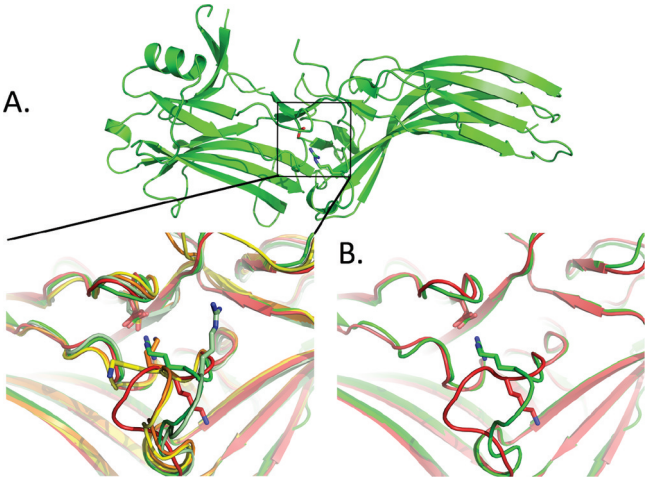

Figure 3. Arg307 is a conformationally variable residue. (A) Arg307 can form a long ion pair with Asp29, which bridges the N- and C-domains of arrestin-2. This interaction is likely weak, as evidenced by the lack of its conservation in arrestin-1 (1CF1 shown in yellow), some crystal forms of arrestin-2 (3GDI shown in green and 3JSY shown in light green), arrestin-3 (3P2D shown in red), and arrestin-4 (1AYR shown in orange), and the multiple orientations observed for the loop containing Arg307 in arrestin-2. Note that homologous residue in arrestin-3, Lys308, points in an opposite direction. (B) A simplified view of the differences between arrestin-2 (green) and arrestin-3 (red).

suggesting that a positive charge in this position could be important.

Next we tested whether reduced c-Raf1 binding of the Arg307Ala mutant affects its ability to promote receptor-dependent ERK1/2 activation. GPCRs activate ERK1/2 via multiple G protein-dependent and independent pathways, one of which involves arrestin scaffolding of c-Raf1−MEK1−ERK1/2 cascade.[32] Although in some cases the pathways can be distinguished by the time course, with rapid phase of ERK1/2 phosphorylation largely mediated by the G-protein and the slower phase attributable to arrestins,[33,34] in other cases both phases appear to be G protein-dependent.[35] An inverse agonist of β2AR, ICI118551, that blocks G protein activation, was

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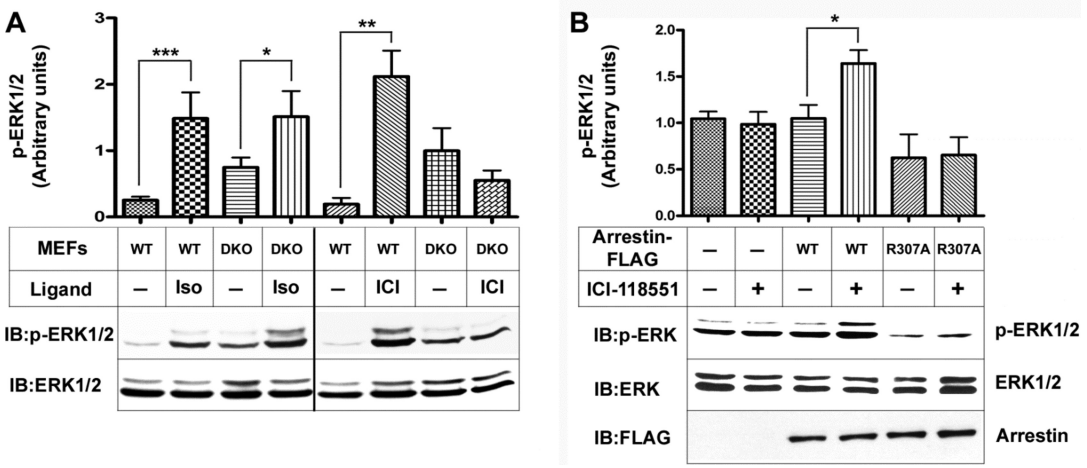

Figure 4. Arg307Ala mutant fails to rescue β2AR-mediated ERK activation in arrestin-2/3 knockout MEFs. (A) WT and arrestin-2/3 double knockout (DKO) MEFs were serum-starved and treated with 1 μM ICI118551 (ICI) or 10 μM isoproterenol (ISO) for 10 min at 37 °C and then lysed, as described in Experimental Procedures. Cell lysates were analyzed by Western using indicated primary antibodies. (B) DKO MEFs were infected with retrovirus encoding GFP (control, −), WT arrestin-2 (WT), or arrestin2-Arg307Ala mutant (R307A). 48 h post-transfection, the cells were serum-starved for 2 h, stimulated with 1 μm ICI118551 for 10 min at 37 °C, lysed, and analyzed by Western blot. Means ± SD of 3−4 independent experiments are shown in bar graphs; representative blots are shown below. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

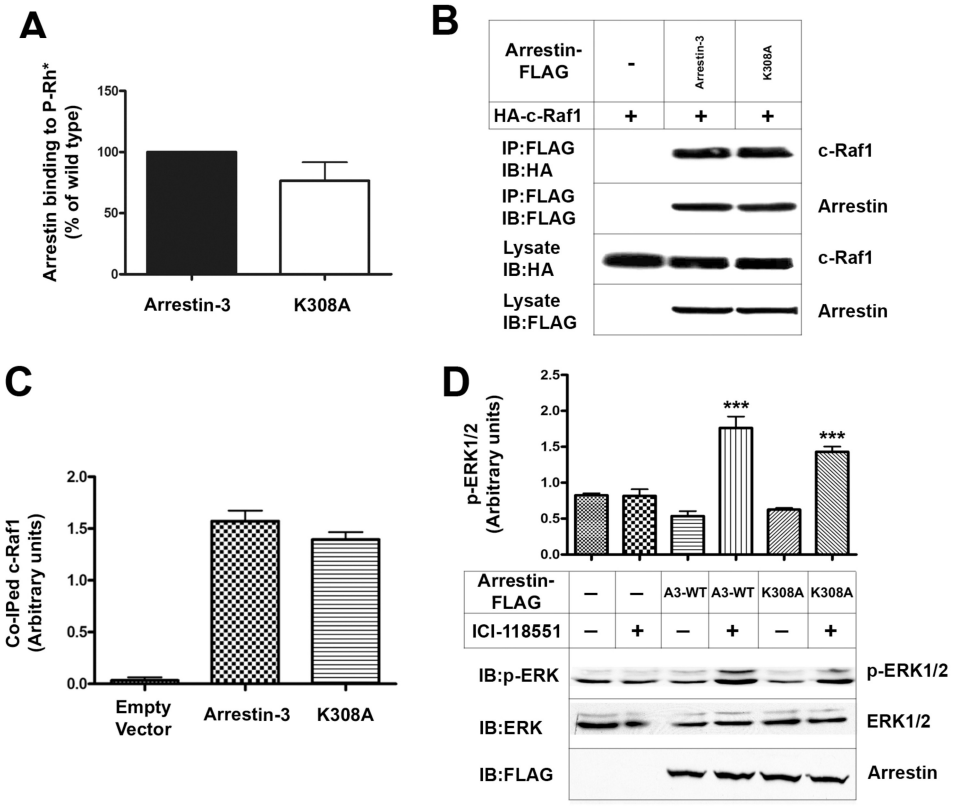

Figure 5. Distinct functional role of homologous positive charge in arrestin-3. (A) The binding of WT arrestin-3 and K308A mutant to P-Rh*. Means ± SD of three experiments performed in duplicate are shown. (B) Flag-tagged WT arrestin-3 and K308A mutant were coexpressed with HA-tagged c-Raf1 in COS7 cells. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immounoprecipitated c-Raf1 was visualized by Western blot with anti-HA antibody. The results of a representative experiment are shown. (C) The intensity of c-Raf1 band in the immunoprecipitate was quantified. Means ± SD of three independent experiments are shown. (D) DKO MEFs were infected with retrovirus encoding GFP (control, −), WT arrestin-3 (A3-WT), or arrestin-3−Lys308Ala mutant (K308A). 48 h post-transfection, the cells were serum-starved for 2 h, stimulated with 1 μm ICI118551 for 10 min at 37 °C, lysed, and analyzed by Western blot. Means ± SD of three independent experiments are shown in bar graphs; representative blots are shown below. $***p < 0.001$.

shown to be a biased ligand, acting as an agonist for nonvisual arrestins.[29] We confirmed this observation by showing that the robust activation of endogenous ERK1/2 induced by ICI118551 via endogenous β2AR is readily detected in WT MEFs, but completely absent in arrestin-2/3 double knockout (DKO) MEFs (Figure 4A). Therefore, we used the ability of

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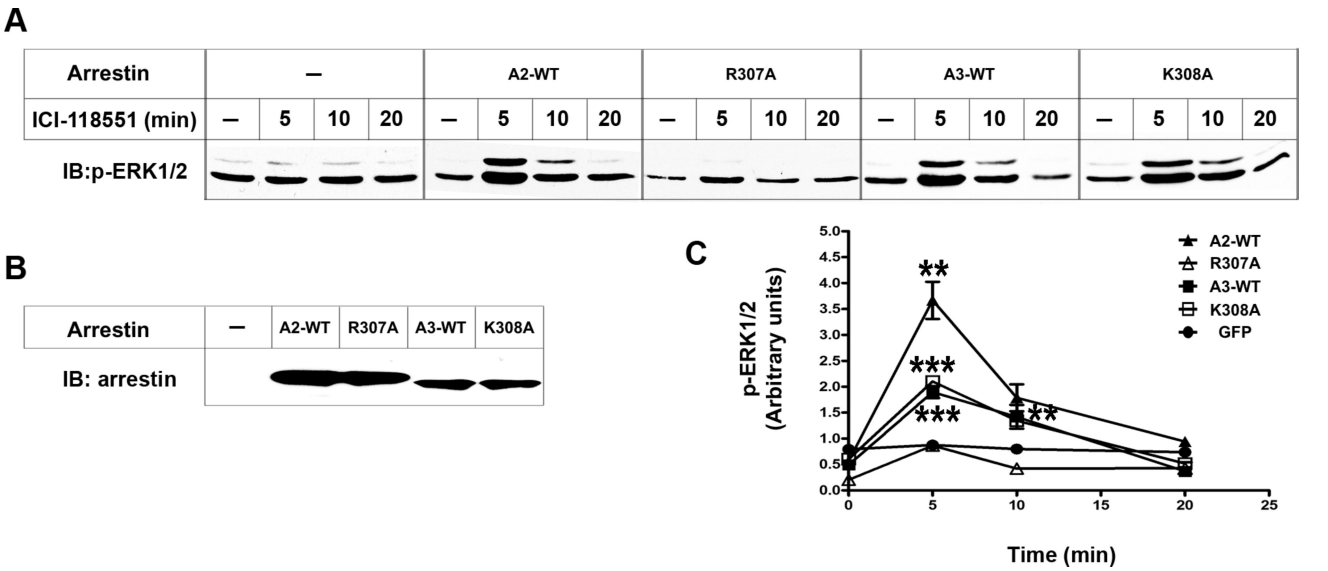

Figure 6. Rapid arrestin-mediated ERK1/2 activation by ICI118551 via β2-adrenergic receptor. (A) DKO MEFs were infected with retrovirus encoding GFP (control), WT arrestin-2 (A2-WT), arrestin-2–Arg307Ala (R307A), WT arrestin-3 (A3-WT), or arrestin-3–Lys308Ala (K308A). 48 h postinfection, DKO-MEFs were serum-starved for 2 h and stimulated with 1 μM ICI118551 for 0, 5, 10, and 20 min at 37 °C. Cells were then lysed and analyzed by Western blot. Representative blot for phospho-ERK is shown. (B) The expression of WT and mutant arrestins was compared by Western blot. Mutants and corresponding parental WT arrestins express at the same level. However, the expression of both forms of arrestin-2 is ~3-fold higher than that of both forms of arrestin-3. (C) Time course of ERK1/2 activation in DKO MEFs expressing GFP (control) or indicated arrestins. Means ± SD of two independent experiments are shown. Statistical significance of the differences (as compared to corresponding zero time point) is shown, as follows: **, $p < 0.01$; ***, $p < 0.001$.

arrestin expressed in DKO MEFs to rescue ICI118551-induced ERK1/2 activation as our assay. We found that WT arrestin-2 successfully rescues ERK1/2 activation by ICI118551, whereas the Arg307Ala mutant expressed at the same level fails to do so (Figure 4B). Thus, reduced c-Raf1 binding by arrestin-2–Arg307Ala (Figure 2A) translates into a complete loss of the ability to productively scaffold the c-Raf1–MEK1–ERK1/2 signaling cascade, suggesting that Arg307 in arrestin-2 plays a critical role in binding c-Raf1 and promoting ERK1/2 activation.

Interestingly, the homologous arrestin-3 residue is a lysine, and Lys308 points in a different direction in the crystal structure of arrestin-3 (Figure 3).[19] To test whether this distinct conformation translates into a different role for this residue in arrestin-3-dependent scaffolding of c-Raf1–MEK1–ERK1/2 module, we constructed an arrestin-3–K308A mutant. We found that this mutation does not significantly affect the ability of arrestin-3 to bind a model receptor, light-activated phosphorhodopsin (Figure 5A). Next, we compared the ability of WT arrestin-3 and its K308A mutant to interact with coexpressed c-Raf1 in COS7 cells and found that both proteins co-immunoprecipitate essentially the same amount of c-Raf1 (Figure 5B,C), suggesting that the role of this positively charged residue in arrestin-3 is different. To test whether the equivalent binding of c-Raf1 translates into an equal ability of arrestin-3 and the K308A mutant to promote the activation of endogenous ERK1/2 in arrestin-dependent manner, we expressed both proteins in arrestin-2/3 DKO MEFs and challenged endogenous β2AR with an arrestin-biased agonist ICI118551 (Figure 5D). We confirmed that DKO MEFs do not show ERK1/2 activation in response to ICI118551. The expression of WT arrestin-3 and its K308 mutant rescued ERK1/2 response to ICI118551 virtually to the same extent (Figure 5D). Thus, Lys308 in arrestin-3 does not play a critical role in c-Raf1 binding and ERK1/2 phosphorylation (Figure 5), in contrast to the homologous Arg307 in arrestin-2 (Figures 2 and 4). These data suggest that even though both nonvisual arrestins scaffold the c-Raf1–MEK1–ERK1/2 cascade, the stereochemical details of their molecular mechanisms are distinct.

To test whether the mutations in nonvisual arrestins change the time course of receptor-dependent ERK1/2 activation, rather than just maximum response, we used DKO MEFS expressing GFP (control), WT arrestin-2, arrestin-2–R307A, WT arrestin-3, or arrestin-3–K308A mutants (Figure 6). We found that in all cases peak ERK1/2 phosphorylation was observed at 5 min of ICI118551 treatment, and by 20 min ERK1/2 activity returned back to basal (Figure 6A,C). In control GFP-expressing DKO MEFs no ERK1/2 activation in response to ICI118551 was detected, once more demonstrating that this effect is strictly arrestin-dependent. Thus, ERK1/2 activation triggered by arrestin-biased β2AR agonist ICI118551 in MEFs is rapid and transient. The time courses confirm that arrestin-2–R307A mutant does not promote ERK1/2 phosphorylation, in contrast to both WT nonvisual arrestins and arrestin-3–K308A mutant. Relatively higher ERK1/2 phosphorylation mediated by WT arrestin-2 likely reflects its ~3-fold higher expression level than that of arrestin-3 (Figure 6B).

DISCUSSION

Nonvisual arrestins facilitate the activation of several MAP kinases in response to GPCR activation, including JNK3,[7] ERK1/2,[8] and p38.[9] Multiple GPCRs have been shown to activate ERK1/2 in an arrestin-dependent manner, including β2AR,[34] angiotensin type 1A receptor,[33] μ-opioid receptor,[36] and the protease-activated receptor-2.[37] Subsequent studies showed that free arrestins also bind ASK1, JNK3, MKK4, c-Raf1, MEK1, and ERK2.[10−12,38−42] Interestingly, while the

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interaction of ASK1, MKK4, and JNK3α2 with free arrestin-3 translates into JNK3 phosphorylation,[10,12] receptor-independent ERK activation by free arrestins has not been observed, possibly due to very low affinity of ERK for free arrestin-2 and -3.[12] Structurally, arrestins are elongated molecules with two cuplike domains.[19,43−46] All identified receptor-binding elements are localized on the concave side of both domains,[13−18,21,27,47] suggesting that binding partners recruited to the arrestin−receptor complex engage the opposite convex surface of the molecule (Figures 1A and 3).

Although arrestin structures suggest that the functions mediated by different surfaces can be modulated independently of each other by specific mutations,[48,49] alanine-scanning mutagenesis of the conserved residues on the nonreceptor-binding side of arrestin-2 revealed numerous mutations that significantly affected receptor binding (Supporting Information Figure S1). Thus, targeted design of arrestins with desired functional characteristics may be a more complex endeavor than previously thought: it appears that the basal conformation of arrestin-2 is stabilized by an intricate network of interactions that spans both the receptor- and kinase-binding surfaces. The functional coupling of these two interfaces needs to be further explored experimentally. Therefore, for the analysis of arrestin-2 interactions with c-Raf1, MEK1, and ERK1/2, we only used ten mutants that demonstrated normal receptor binding (Figure 1B), indicative of proper folding. Nine of these showed essentially WT interactions with all three kinases in the c-Raf1−MEK1−ERK1/2 pathway, whereas c-Raf1 binding was selectively impaired by the Arg307Ala mutation (Figure 2). Although these data do not mean that Arg307 is the only residue involved in c-Raf1 interaction, it appears to be an important docking point for this kinase.

Arg307 is located at the interdomain interface, and in most structures of arrestin-2 interacts with Asp29 forming an interdomain bridge (Figure 3). This interaction is quite long, ranging from 3.5 to 5.8 Å, and likely contributes little energy to the crystallographically observed conformations. In arrestin-2 structures G4M,[44] G4R,[44] 1ZSH,[50] and 3GDI[51] the interaction is present, but it is absent in 3GC3[51] and 1JSY.[45] The interaction is observed in 1AYR, the only arrestin-4 structure.[46] Arrestin-1 and -3 do not form this interaction in the crystals, although Arg307 is substituted by lysine in arrestin-3 and arrestin-1, preserving positive charge.[6] Our observation that Arg307Ala is impaired in c-Raf1 binding and unable to promote detectable ERK1/2 activation indicates that in the c-Raf1 bound state Arg307 interacts with c-Raf1, rather than with Asp29. Arg307 is localized on the periphery of the interface between the N- and C- domains (Figure 3). Its interaction with Asp29 can bridge the two domains, but it is likely one of many weak interactions that stabilize the basal arrestin conformation and are broken upon receptor binding.[52]

We showed that ERK1/2 activation via endogenous β2AR stimulated by ICI118551 in MEFs is strictly arrestin-dependent (Figure 4A). Using this model, we demonstrated that impaired c-Raf1 binding results in the inability of the Arg307Ala mutant to scaffold productively the c-Raf1−MEK1−ERK1/2 cascade (Figure 4B), despite its normal ability to bind receptor, MEK1, and ERK2 (Figures 1 and 2). Interestingly, Arg307Ala shows a tendency to act as a dominant-negative mutant, reducing overall ERK1/2 activity in MEFs (Figure 4B), likely via sequestering MEK1 and/or ERK1/2, both of which bind normally to this mutant (Figure 2B,C). Thus, as far as ERK1/2 activation is concerned, arrestin-2−Arg307Ala is the first signaling-biased arrestin constructed. Importantly, this function of arrestin-2 was selectively suppressed by a point mutation, which did not appreciably affect arrestin-2 binding to MEK1, ERK2, or receptor. However, we did not test many other reported arrestin-2 functions, so it is conceivable that its interactions with some other partners were also affected.

The analysis of the time course of arrestin-mediated ERK1/2 activation via endogenous β2AR stimulated by ICI118551 in MEFs shows that WT arrestin-2 and arrestin-3, as well as arrestin-3−Lys308Ala mutant, comparably rescue ERK1/2 activation at all time points tested, whereas arrestin-2−Arg307Ala is consistently inactive (Figure 6). Thus, the differences between arrestin-2−Arg307Ala and arrestin-3−Lys308Ala mutants to facilitate ERK1/2 phosphorylation at 10 min (Figures 4 and 5) reflect their inherent activity, rather than different kinetics of the response. Interestingly, we found that ERK1/2 activation by ICI118551 in MEFs, which we showed to be strictly arrestin-mediated (Figure 4A), is transient: the response reaches the peak at 5 min and rapidly declines, returning to the basal level by 20 min (Figure 6). ERK can be activated by GPCRs via distinct G protein- and arrestin-mediated mechanisms.[32] Several previous studies using overexpressed angiotensin II,[33] β2AR,[34] and parathyroid hormone receptors[53] suggested that ERK activation via G proteins is rapid and transient, whereas arrestin-mediated ERK1/2 activation is slow but prolonged. However, ERK1/2 activation by endogenous M3 muscarinic receptor via Gq in HEK293 cells was recently shown to be as long-lasting as previously reported arrestin-mediated activation.[35] We found that arrestin-mediated ERK1/2 phosphorylation in MEFs is rapid and fades away in less than 20 min (Figure 6), i.e., essentially as quickly as previously reported G protein-mediated ERK1/2 activation in other cell types. Importantly, in our experiments ERK1/2 was activated in response to the stimulation of β2AR, the same receptor that was previously reported to induce prolonged arrestin-mediated ERK1/2 activation in HEK293 cells.[34] Previous studies used four different GPCRs,[33−35,53] so that distinct kinetics of G protein-mediated ERK1/2 activation could be explained by the use of different receptors.[54] However, here we used the same β2AR as Shenoy et al.,[34] yet found very different timing of arrestin-mediated ERK1/2 phosphorylation (Figure 6). The fact that we activated ERK1/2 via endogenous β2AR expressed at fairly low level, whereas Shenoy et al.[34] overexpressed WT and mutant β2AR could have contributed to this difference. Conceivably, cellular context also affects the time course of ERK1/2 activation via a particular pathway at least as much as the subtype of activated GPCR.

Our finding that alanine substitution of the homologous positively charged residue in arrestin-3, Lys308, does not affect the ability of this subtype to bind c-Raf1 and promote ERK1/2 activation (Figure 5) is the first demonstration that when both nonvisual arrestins perform the same function, the two subtypes employ distinct molecular mechanisms. Structurally, several elements in arrestins appear to be fairly flexible, assuming distinct conformations not only in different arrestin subtypes, but even in different monomers within crystal oligomer.[19,43,44] Therefore, these differences are often dismissed as mere indication of the plasticity of certain elements in the protein. However, our data suggest that subtle structural differences between arrestin-2 and -3 (Figure 3)[19] revealed by the crystal structures can have significant functional consequences.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: strategy and primers used for arrestin-2 and -3 mutagenesis by PCR; Table S2: cell-free expression yields, protein stability, and P-Rh* binding of arrestin-2 mutants; Figure S1: P-Rh* binding of arrestin-2 and arrestin-3 mutants used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 615-322-7070. Fax: 615-343-6532. E-mail: vsevolod.gurevich@vanderbilt.edu.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; ERK, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; MEK1, dual specificity mitogen-activated protein kinase 1, encoded by the MAP2K1 gene in humans; JNK3, c-Jun N-terminal kinase; c-Raf1, aka c-Raf, proto-oncogene serine/threonine-protein kinase encoded in humans by the RAF1 gene.

■ ADDITIONAL NOTE

[a]We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "arrestin 3" in HUGO database).

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