The Third Intracellular Loop of α2-Adrenergic Receptors Determines Subtype Specificity of Arrestin Interaction*

Jessica L. DeGraff‡, Vsevolod V. Gurevich§, and Jeffrey L. Benovic‡

From the ‡Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the §Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

Nonvisual arrestins (arrestin-2 and -3) serve as adaptors to link agonist-activated G protein-coupled receptors to the endocytic machinery. Although many G protein-coupled receptors bind arrestins, the molecular determinants involved in binding remain largely unknown. Because arrestins selectively promote the internalization of the α2b-, and α2c-adrenergic receptors (ARs) while having no effect on the α2aAR, here we used α2ARs to identify molecular determinants involved in arrestin binding. Initially, we assessed the ability of purified arrestins to bind glutathione S-transferase fusions containing the third intracellular loops of the α2aAR, α2bAR, or α2cAR. These studies revealed that arrestin-3 directly binds to the α2bAR and α2cAR but not the α2aAR, whereas arrestin-2 only binds to the α2cAR. Truncation mutagenesis of the α2aAR identified two arrestin-3 binding domains in the third intracellular loop, one at the N-terminal end (residues 194–214) and the other at the C-terminal end (residues 344–368). Site-directed mutagenesis further revealed a critical role for several basic residues in arrestin-3 binding to the α2bAR third intracellular loop. Mutation of these residues in the holo-α2cAR and subsequent expression in HEK 293 cells revealed that the mutations had no effect on the ability of the receptor to activate ERK1/2. However, agonist-promoted internalization of the mutant α2bAR was significantly attenuated as compared with wild type receptor. These results demonstrate that arrestin-3 binds to two discrete regions within the α2bAR third intracellular loop and that disruption of arrestin binding selectively abrogates agonist-promoted receptor internalization.

G protein-coupled receptors (GPCRs) transduce extracellular stimuli into intracellular signaling via coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins) (1). To ensure that stimuli are translated into signals of appropriate magnitude and specificity, these signaling cascades are tightly regulated. GPCRs are subject to three principal modes of regulation: desensitization, in which a receptor becomes refractory to continued stimuli; endocytosis, whereby receptors are removed from the cell surface; and down-regulation, in which total cellular receptor levels are decreased (2, 3). Although multiple mechanisms contribute to these regulatory processes, GPCR phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent binding of arrestins plays an important role in the regulation of many GPCRs (2).

Four mammalian arrestins have been identified with arrestin-1 and -4 being specific to the visual system and arrestin-2 and -3 (also termed β-arrestin-1 and -2) being ubiquitously expressed (4–7). Arrestin binding to activated-phosphorylated GPCRs results in the physical uncoupling of receptor from G protein, a process that functions to terminate agonist-mediated signaling. The two nonvisual arrestins also directly interact with clathrin (8), the adaptor protein AP2 (9), and phosphoinositides (10) to promote GPCR internalization. Indeed, nonvisual arrestins have been implicated in the desensitization and internalization of a wide variety of GPCRs including members of the class 1 (rhodopsin-like) and class 2 (secretin-like) families (2, 11). Nevertheless, despite numerous studies that demonstrate an essential role for arrestins in the regulation of GPCR signaling and trafficking, the precise molecular determinants within GPCRs required for arrestin binding have not been thoroughly characterized.

Three α2-adrenergic receptors, α2aAR, α2bAR, and α2cAR, have been identified (12–14). α2ARs are activated by the catecholamines epinephrine and norepinephrine and regulate sympathetic outflow and cardiovascular function in vivo (15–17). All three α2AR subtypes primarily couple to the Gi family of G proteins and modulate a variety of signaling pathways including activation of phospholipase A2 (18), phospholipase D (19), and extracellular regulated kinases ERK1/2 (20–22) and inhibition of adenylyl cyclase (23). α2AR signaling is also subject to dynamic regulation. The α2aAR and α2cAR are subject to agonist-dependent phosphorylation by GRKs (24). Moreover, previous studies have revealed a role for arrestins in agonist-promoted internalization of α2ARs with arrestin-3 promoting internalization of the α2bAR and α2cAR and arrestin-2 selectively promoting internalization of the α2aAR. Interestingly, α2aAR internalization was not promoted by either arrestin, suggesting arrestin/receptor binding specificity (25).

Previous studies have demonstrated an important role for the third intracellular loops of the α2ARs in mediating protein-protein interaction. These loops are quite large (>150 amino acids) and include sites for GRK phosphorylation (26), Gi activation (27), and binding of 14-3-3 (28), spinophilin (29), and arrestin (30). Although arrestin binding to GPCRs is dependent on both the phosphorylation and activation state of the receptor (31), the receptor domains that mediate the agonist-dependent interaction...
nature of arrestin binding have not been thoroughly characterized. Because third intracellular loops mediate agonist-dependent binding and activation of heterotrimeric G proteins (32, 33), it seems likely that specific regions of the third intracellular loop might also confer the agonist dependence and selectivity of arrestin binding. Indeed, the third intracellular loop has been implicated in arrestin interaction for a number of GPCRs; including rhodopsin (34), α2-αd-adrerenergic (30), M3, muscarinic (30), δ-iodopitryramineα3 (36), CXC4R4 (37), and the luteinizing hormone/choriogonadotropin (LH/CG) receptor (38).

The subtype-specific differences in arrestin sensitivity for the three α2ARs provides a useful model to identify specific regions involved in arrestin binding. To address this issue, we studied arrestin binding to a series of glutathione S-transferase (GST) fusion proteins containing various regions of the third intracellular loops of the three α2AR subtypes. Our results revealed arrestin binding specificity that recapitulates the arrestin specificity observed previously in α2AR trafficking (25). Truncation and site-directed mutagenesis revealed that arrestin-3 binds to two discrete regions within the α2AR third intracellular loop. Moreover, disruption of arrestin binding selectively abrogated agonist-promoted internalization of the α2AR. These studies help to address questions of specificity in GPCR/arrestin interaction that ultimately will lead to a better understanding of the role of arrestins in regulating receptor-mediated signaling.

EXPERIMENTAL PROCEDURES

Plasmid Construction—FLAG-tagged α2aAR, α2bAR, and α2cAR were cloned into pDNA3 as described previously (25). The third intracellular loops of the α2AR (residues 218–374), α2AR (residues 194–368), and α2bAR (residues 232–379) were ampicillin-resistant derivatives with full-length receptors as template. The PCR products were cut with EcoRI/XhoI (α2AR and α2bAR) or EcoRI/Sall (α2cAR), gel-purified, inserted into the plasmid pGEX4T-2 in-frame with GST, and sequenced. The following α2AR third intracellular loop truncation mutants were made and cloned into pGEX4T-2 using the same strategy: N-terminal (NT) (residues 194–292), NT2 (194–292), NT2 (206–292), C-terminal (CT) (293–368), CT2 (293–358), and CT3 (344–368), respectively, and sequenced. The oligonucleotide-directed PCR mutagenesis was constructed by oligonucleotide-directed PCR mutagenesis in either the NT1 construct (K200A, R201A, K202A, R203A, N205A, R206A, R207A, R208A), the CT3 construct (L344A/L345A, R347A, Q355A, W356A/W357A, R358A, R359A, R355A, W353A, R357A, R358A, R359A, R360A, R362A, R364A, R365A, R366A, K367A, and R368A), or the third loop construct (K204A/K205A (KR), R204A/R205A (RR), K204A/K205A, R204A/R205A, and R204A/R205A) were sequenced. A C-terminal fragment of the mutant α2AR was generated using a mutagenic sense primer and an Sp6 antisense primer, and a C-terminal fragment of the mutant α2AR was inserted into the plasmid pGEX4T-2 in-frame with GST, and sequenced. The following α2AR third intracellular loop truncation mutants were made and cloned into pGEX4T-2 using the same strategy: N-terminal (NT) (residues 194–292), NT2 (194–292), NT2 (206–292), C-terminal (CT) (293–368), CT2 (293–358), and CT3 (344–358) were sequenced. The following α2AR third intracellular loop truncation mutants were made and cloned into pGEX4T-2 using the same strategy: N-terminal (NT) (residues 194–292), NT2 (194–292), NT2 (206–292), C-terminal (CT) (293–368), CT2 (293–358), and CT3 (344–358) were sequenced.

RESULTS AND DISCUSSION

Analysis of Phospho-ERK1/2—HEK 293 cells in 60-mm dishes were transfected as described above and then split into three wells of a poly-l-lysine-coated 12-well dish. The following day, cells were serum-starved overnight in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum. The next day, cells were stimulated with 10 μM of FuGENE-6 reagent according to the manufacturer’s protocol. Briefly, cells were incubated with the FuGENE-DNA mixture for 5 h and then split into poly-l-lysine-coated 12-well dishes (for ERK1/2 assays) or 24-well dishes (for enzyme-linked immunosorbent assay). Enzyme-linked immunosorbent assays were performed 24 h after transfection as described previously (25), whereas ERK1/2 assays were done 48 h after transfection.

Expression and Purification of GST-α2AR Fusion Proteins—BL21 (DE3) lys cells transformed with a GST-α2AR fusion construct were grown overnight at 37 °C, diluted 1:100 into LB containing ampicillin, grown for 3 h at 37 °C, and then induced with 0.1 mM isopropyl-thiogalactopyranoside for 2 h at 30 °C. Cells were pelleted (3000 × g for 30 min) and washed with phosphate-buffered saline (PBS) containing 1 mM dithiothreitol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml benzamidine, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). Cells were pelleted, resuspended in 1–3 ml of PBS plus protease inhibitors (per 100 ml of culture), lysed by sonic disruption for 10 min, and then centrifuged for 10 min at 10,000 rpm. The supernatant was then aliquoted (0.4 ml), frozen, and stored at −80 °C until needed. Aliquots were thawed on ice, Triton X-100 (2% final) and sarcosyl (0.5% final) were added, and the cells were frozen, thawed, and centrifuged for 1 h (30,000 rpm in TLA-45 rotor). The supernatant (~200 μl) was then incubated with 200 μl of 50% glutathione-agarose bead slurry for 1 h at 4 °C. The beads were washed twice with PBS containing protease inhibitors and 1% Triton X-100 and resuspended in 200 μl of PBS with protease inhibitors. To quantify protein amounts, 20 μl of resuspended beads were incubated with SDS sample buffer and centrifuged, and the supernatant was electrophoresed on a 10% SDS-polyacrylamide gel. The gels were stained with Coomassie Blue, and protein levels were quantified using bovine serum albumin as standard.

Antennas Differentially Bind to α2AR Third Intracellular Loops—Previous studies have demonstrated subtype-specific differences in arrestin-promoted internalization of α2ARs with arrestin-3 enhancing internalization of α2AR and α2bAR and arrestin-2 only acting on the α2AR (25). To explore the mechanism of arrestin binding selectivity for α2ARs, we initially focused on the third intracellular loop of the α2ARs. The first and second intracellular loops of the α2ARs share sequence homology; however, the third intracellular loops have very divergent sequences, and this region likely directly confers the α2AR-specific structure-function properties.

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**α₂AR Third Loop Determines Arrestin Interaction**

α₂AR third loop, and arrestin-3 bound to both the GST-α₂AR and the GST-α₂AR third loops but not to the α₂AR (Fig. 1B). A dose-response analysis was next performed to determine whether there were binding differences between arrestin-2 and -3 and the α₂AR. Arrestin-3 was found to bind much more effectively to the α₂AR as compared with arrestin-2 with ~20-fold more binding at the highest concentrations of arrestin (Fig. 1C). Overall, these results largely recapitulate the selectivity of arrestins in promoting internalization of the α₂ARs (25) and suggest that this selectivity is mediated by differences in arrestin binding to the third intracellular loops of these receptors.

Identification of Arrestin-3 Binding Domains within the α₂bAR Third Loop—In an effort to identify specific arrestin binding domains, we further investigated the interaction of arrestin-3 with the α₂bAR third loop. Initially, the third loop was bisected into NT (residues 194–292) and CT (residues 293–368) pieces and tested for arrestin binding (Fig. 2A). These studies revealed that both the NT and CT regions of the α₂bAR third loop bind arrestin-3, albeit not as well as the intact third loop (Fig. 2B). Truncation mutagenesis of the NT construct revealed that arrestin-3 binding was primarily localized to the first ~20 residues as NT1 (residues 194–214) bound arrestin-3 as well as NT, whereas NT2 (residues 206–292) did not bind (Fig. 2B). Similar analysis of CT revealed that arrestin-3 binding was primarily localized to the last ~25 amino acids since CT3 (residues 344–368) bound arrestin-3, whereas CT1 (residues 293–312), which contains a long stretch of acidic residues implicated previously in receptor desensitization (39), and CT2 (residues 313–358) did not bind (Fig. 2B). These results suggest that the proximal and distal ends of the third intracellular loop of the α₂bAR contain the major arrestin-3 binding domains.

To define specific residues within NT1 and CT3 that contribute to arrestin binding, a series of alanine point mutants were generated and tested for their ability to bind arrestin-3. Point mutations introduced into the NT1 and CT3 are indicated by an asterisk in Fig. 3, A and B, respectively, whereas double mutations are underlined. These studies revealed that mutation of basic residues within the NT1 and CT3 constructs resulted in a dramatic reduction in arrestin-3 binding. Specifically, mutation of Arg-201, Arg-204, and Arg-205 in NT1 effectively disrupted arrestin-3 binding, whereas mutation of Arg-207 partially disrupted binding (Fig. 3A). In contrast, mutation of Lys-200, Ser-202, Asn-203, Pro-205, and Arg-206 in NT1 had no significant effect on arrestin-3 binding. The placement of essential residues involved in arrestin binding was interesting given recent studies demonstrating that mutation of Lys-382 in the third loop of the parathyroid hormone receptor reduced arrestin-promoted internalization (40), whereas residues in the LH/CG receptor important for arrestin-promoted internalization are localized within the analogous region of the third intracellular loop (38). Mutational analysis of CT3 also revealed the involvement of basic residues in arrestin binding with mutation of several arginines (358, 359, 360, 365, and 368) as well as Lys-367, resulting in a significant reduction...
in arrestin-3 binding (Fig. 3B). In contrast, mutation of Leu-344 and -345 and Gln-355 had a partial effect on binding, whereas Gln-362, Thr-364, and Glu-366 mutations had no effect on arrestin-3 binding. The important role of the proximal and distal ends of the third intracellular loop in arrestin binding is reminiscent of the domains implicated in G protein binding and activation (27). In fact, previous studies have identified a BBXXB motif (where B is a basic residue and X is any residue) in the α2AR involved in Gi activation with Lys-367 and Arg-368 contributing to this motif (27, 32). This suggests that significant overlap between the arrestin-3 and Gi binding sites on the α2AR will likely contribute to the mechanism by which arrestin mediates desensitization (i.e. G protein uncoupling).

To verify that specific arrestin binding mutations made within the NT1 and CT3 constructs were important for arrestin binding in the context of the whole third loop, a series of GST-α2AR third loop mutant proteins were generated. Four different GST-α2AR fusions incorporating either the K200A/R201A (KR), R204A/R205A (RR), R358A/R359A/R360A (3R), or RR/3R mutations were used in binding assays with purified arrestin-3 and compared with the wild type third loop. The ability of the KR and 3R mutants to bind arrestin-3 was modestly reduced as compared with the wild type α2AR (Fig. 4A). However, the RR and RR/3R mutant binding to arrestin-3 was strongly attenuated with an 80–90% reduction in binding. To detect potential binding differences between the RR and RR/3R mutants, a dose-response analysis was performed. The RR mutation alone had a very similar binding pattern to RR/3R with the R204A/R205A mutation almost completely disrupting arrestin-3 binding to the α2AR third loop (Fig. 4B). Taken together, these results suggest that the α2AR third intracellular loop contains two arrestin-3 binding domains with the N-terminal region playing an 80% reduction in binding. To detect potential binding differences between the RR and RR/3R mutants, a dose-response analysis was performed. The RR mutation alone had a very similar binding pattern to RR/3R with the R204A/R205A mutation almost completely disrupting arrestin-3 binding to the α2AR third loop (Fig. 4B). Taken together, these results suggest that the α2AR third intracellular loop contains two arrestin-3 binding domains with the N-terminal region playing an important role of the proximal and distal ends of the third intracellular loop in arrestin binding is reminiscent of the domains implicated in G protein binding and activation (27). In fact, previous studies have identified a BBXXB motif (where B is a basic residue and X is any residue) in the α2AR involved in Gi activation with Lys-367 and Arg-368 contributing to this motif (27, 32). This suggests that significant overlap between the arrestin-3 and Gi binding sites on the α2AR will likely contribute to the mechanism by which arrestin mediates desensitization (i.e. G protein uncoupling).

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**Internalization of Wild Type and Mutant α2ARs in HEK 293 Cells**—We next incorporated the various third loop mutations (KR, RR, 3R, and RR/3R) into the holo-α2AR. Because arrestins are involved in agonist-promoted internalization of the α2AR (25), we anticipated that disrupting arrestin binding to the α2AR third intracellular loop would attenuate receptor internalization. HEK 293 cells expressing FLAG-tagged wild type or mutant α2ARs were incubated with agonist for 30 min and then analyzed for cell surface receptors by enzyme-linked immunosorbent assay (Fig. 5). Internalization of the wild type α2AR was ~30% after agonist treatment, consistent with previous studies of α2AR internalization in HEK 293 cells (41). Internalization of the KR and 3R mutant receptors was very similar to that of the wild type α2AR, consistent with the in vitro data showing that these mutations did not severely disrupt arrestin binding. In contrast, internalization of the RR mutant was reduced ~50%, whereas the RR/3R mutant was decreased ~65% as compared with the wild type receptor. These data suggest that disrupting arrestin binding to the third intracellular loop of the α2AR has an inhibitory effect on agonist-promoted receptor internalization. These results also help to confirm the important role of arrestins in mediating internalization of the α2AR.

**Signaling of Wild Type and Mutant α2ARs in HEK 293 Cells**—To ensure that the various mutations did not directly affect signaling of the α2AR, we next analyzed the ability of the wild type and mutant α2ARs to activate ERK1/2. Our previous studies demonstrated that all three α2AR subtypes activate ERK1/2 in an agonist-dependent manner via a pathway that is Gi- and Ras-dependent but arrestin- and internalization-independent (25). HEK 293 cells expressing wild type or mutant (RR, 3R, RR/3R) α2ARs were incubated with agonist...
for 0, 5, or 30 min and then analyzed for ERK activation by immunoblotting for phospho-ERK1/2. All receptors activated ERK1/2 from 5- to 7-fold after a 5-min treatment with agonist, suggesting that the mutations that inhibit arrestin binding and receptor internalization have no significant effect on \( \alpha_2 \)AR activation of signaling (Fig. 6, A and B).

**Conclusions**—Our results on the NT1 region of the \( \alpha_2 \)AR suggest the importance of a BXXBB binding motif that is essential for arrestin-3 binding. Mutation of Arg-201, Arg-204, or Arg-205 completely disrupted arrestin-3 binding, whereas mutation of surrounding residues had minimal effect on arrestin binding (Fig. 3). Interestingly, the analogous region of the \( \alpha_2 \)AR, but not the \( \alpha_2 \)AR, can also directly bind arrestin-3 (data not shown). Although the three \( \alpha_2 \)ARs share significant homology within the N-terminal 20 residues of the third intracellular loops, a key residue present in both the \( \alpha_2 \)AR (Arg-201) and the \( \alpha_2 \)AR (Arg-234) is replaced with Gln-221 in the \( \alpha_2 \)AR (Fig. 7). The absence of this particular basic residue within the \( \alpha_2 \)AR may disrupt arrestin binding. It is also interesting to note that mutation of Arg-239 (the last B in the BXXBB motif) within the NT1 region of the \( \alpha_2 \)AR completely disrupts arrestin-3 binding (data not shown), further establishing the importance of basic residues within this region for arrestin binding. Several recent studies have also suggested a role for receptor third intracellular loops in arrestin binding (30, 34–38). For example, the third loops of the \( \delta \)-opioid receptor (35) and the LH/CG receptor (38) can directly bind arrestins, and third loop peptides from the LH/CG receptor inhibit receptor desensitization by sequestering arrestin-2 (38). The domains involved in these interactions share similar homology and generally contain several basic residues as well as serines and/or threonines (Fig. 7). Although the \( \delta \)-opioid receptor studies suggested a role for two serines in arrestin binding (35), all of these domains contain the BXXBB motif and help to establish the importance of such a motif in arrestin binding.

At least two sites within arrestin are involved in GPCR binding, a phosphorylation recognition site that binds to phosphoserines/threonines on the receptor and an activation recognition site that binds the agonist-bound conformation of the receptor (31). Arrestin binding to phosphoserines/threonines is thought to destabilize the arrestin polar core and promote secondary high affinity binding to the receptor and binding to the phospholipid bilayer (42). In our studies, the third loops were not phosphorylated; however, arrestin binding sites that would normally be inaccessible in the unactivated holo-receptor are likely available for arrestin binding in the isolated third intracellular loops. One possible reason why the \( \alpha_2 \)AR binds arrestin-2 and -3 with higher affinity than the \( \alpha_2 \)AR may be the acidic stretch of amino acids located within the \( \alpha_2 \)AR third loop. This acidic stretch is involved in mediating receptor desensitization (39) and may act to destabilize the arrestin polar core and promote binding much like phosphorylation does.

It is important to note that although the RR and RR3R mutant \( \alpha_2 \)ARs demonstrated a defect in agonist-promoted receptor internalization, the inhibition was not complete. This suggests either the incomplete disruption of arrestin binding within the third loop (as seen in our GST binding studies) or a role for additional arrestin binding sites on the receptor. Additional potential arrestin binding sites on \( \alpha_2 \)ARs include the second intracellular loop, which contains the DRY motif that has been implicated in arrestin association in several GPCRs (43–45). Further studies involving \( \alpha_2 \)AR domains may reveal insight as to how arrestin regulates both internalization and signaling of receptors as recent studies propose that arrestin binding to different regions of a receptor can regulate different functions (40).

In summary, we have identified two discrete arrestin-3 binding domains within the \( \alpha_2 \)AR third intracellular loop. Specifically Arg-201, Arg-204 and Arg-205, and to a lesser extent...
Arg-358, Arg-359, and Arg-360, are important for arrestin binding in vitro. Mutation of these residues severely impairs agonist-dependent receptor internalization, suggesting the critical nature of arrestin in this process. Interestingly, arrestin-3 directly interacts with the basic residues of the G protein activation motif located in the third loop, suggesting a model by which arrestin binding displaces G protein, thus attenuating signaling in addition to linking the receptor to clathrin-coated pits.

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Jessica L. DeGraff, Vsevolod V. Gurevich and Jeffrey L. Benovic

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