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Arrestin-2 Interacts with the Ubiquitin-Protein Isopeptide Ligase Atrophin-interacting Protein 4 and Mediates Endosomal Sorting of the Chemokine Receptor CXCR4*

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The chemokine receptor CXCR4 is rapidly targeted for lysosomal degradation by the E3 ubiquitin ligase atrophin-interacting protein 4 (AIP4). Although it is known that AIP4 mediates ubiquitination and degradation of CXCR4 and that perturbations in these events contribute to disease, the mechanisms mediating AIP4-dependent regulation of CXCR4 degradation remain poorly understood. Here we show that AIP4 directly interacts with the amino-terminal half of nonvisual arrestin-2 via its WW domains. We show that depletion of arrestin-2 by small interfering RNA blocks agonist-promoted degradation of CXCR4 by preventing CXCR4 trafficking from early endosomes to lysosomes. Surprisingly, CXCR4 internalization and ubiquitination remain intact, suggesting that the interaction between arrestin-2 and AIP4 is not required for ubiquitination of the receptor at the plasma membrane but perhaps for a later post-internalization event. Accordingly, we show that activation of CXCR4 promotes the interaction between AIP4 and arrestin-2 that is consistent with a time when AIP4 co-localizes with arrestin-2 on endocytic vesicles. Taken together, our data suggest that the AIP4·arrestin-2 complex functions on endosomes to regulate sorting of CXCR4 into the degradative pathway.

The chemokine receptor CXCR4, a G protein-coupled receptor (GPCR),[3] together with its cognate ligand, stromal cell-derived factor-1α, also termed CXCL12, play an important role in several biological processes such as development of the heart and brain, leukocyte chemotaxis, and stem cell homing (1–3). Although CXCR4 dysregulation has been linked to several pathologies, especially cancer, the molecular mechanisms regulating CXCR4 remain poorly understood (4, 5). Activated CXCR4 is targeted for lysosomal degradation through a pathway involving ubiquitination of carboxyl-terminal tail lysine residues mediated by the E3 ubiquitin ligase atrophin-interacting protein 4 (AIP4) (6, 7). AIP4 belongs to the neural precursor cell-expressed developmentally down-regulated gene 4-like family of homologous to E6-AP carboxyl-terminal domain E3 ubiquitin ligases, which interact with their target proteins either directly or indirectly via their WW domains or possibly other domains (8, 9).

In addition, AIP4 regulates endosomal sorting of activated CXCR4 by targeting the receptor to the endosomal sorting complex required for transport pathway (7), which is a complex network of proteins that recognize and sort ubiquitinated cargo into the multivesicular body (10). Entry into this pathway requires the action of HRS, a ubiquitin-binding protein localized to flat clathrin lattices on endosomes adjacent to invaginating domains, where it sequesters ubiquitinated cargo destined for entry into the multivesicular body for subsequent degradation (11, 12). CXCR4 and AIP4 localize to HRS-positive microdomains on endosomes (7). In addition, AIP4 mediates CXCR4-dependent ubiquitination of HRS, an action that likely plays a role in the sorting function of HRS (7). Whether additional proteins play a role in the endosomal sorting of CXCR4, however, remains to be determined.

Nonvisual arrestins, arrestin-2 and arrestin-3 (also termed β-arrestin-1 and β-arrestin-2, respectively), function in GPCR desensitization and also serve as endocytic adaptors to recruit phosphorylated receptors for internalization through clathrin-coated pits (13). Arrestins have also been implicated in ubiquitination of GPCRs. Ubiquitination of the β2-adrenergic receptor appears to require arrestin-3 and a yet to be identified E3 ubiquitin ligase (14). Arrestins have also been implicated in CXCR4 desensitization (15, 16) and are thought to play a role in CXCR4 internalization (15, 17), but whether they function in ubiquitination and degradation of activated CXCR4 remains unknown. Therefore, to gain further insight into the molecular mechanisms mediating CXCR4 down-regulation, we examined the role of nonvisual arrestins in targeting CXCR4 to the degradative pathway.

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; AIP4, atrophin-interacting protein 4; GST, glutathione *S*-transferase; E3, ubiquitin-protein isopeptide ligase; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HA, hemagglutinin; WCL, whole cell lysates; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; IP, immuno precipitation.

EXPERIMENTAL PROCEDURES

Cell Lines, cDNA Constructs, Antibodies, and siRNA—HEK293 (Microbix, Toronto, Canada) and HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA) supplemented with fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA). HA-tagged CXCR4 in pcDNA3 and FLAG-tagged ubiquitin and AIP4 in pCMV-10 were as previously described (7). For GST fusion constructs, full-length AIP4 (amino acids 2–862) and WW domains I–IV (amino acids 260–486) were amplified by PCR and cloned into pGEX-4T2. HA-CXCR4 was amplified by PCR and subcloned into the EcoRI and ApaI sites of pEYFP-N1 (Clontech, Mountain View, CA). Full-length AIP4 was amplified by PCR and subcloned into the KpnI and BamHI sites of a plasmid encoding monomeric Cherry (pmCh), kindly provided by Dr. R. Y. Tsien (Howard Hughes Medical Institute, University of California, San Diego) (18). Arrestin-2 was subcloned into the XhoI and ApaI sites of pEYFP-C1. All of the constructs were sequenced to verify that no errors were introduced by the PCR. GST fusion constructs of the four individual AIP4 WW domains were as described (19) and kindly provided by Dr. Tony Pawson (Mount Sinai Hospital, Toronto, Canada). The anti-CXCR4 rat monoclonal antibody was as previously described (6). The anti-HA monoclonal and polyclonal antibodies were from Covance (Berkeley, CA). The anti-FLAG M2 and M2-HRP monoclonal antibodies and FLAG polyclonal antibody were from Sigma-Aldrich. The anti-β-tubulin monoclonal antibody was from Accurate Chemical and Scientific (Westbury, NY). The anti-actin monoclonal antibody was from MP Biomedicals (Aurora, OH). The anti-EEA1 mouse monoclonal antibody was from BD Biosciences (San Jose, CA). The anti-LAMP2 (H4B4) antibody was from the Developmental Studies Hybridoma Bank at the University of Iowa. Alexa 594-conjugated anti-mouse antibody was from Invitrogen. The anti-AIP4 mouse monoclonal (G11) and goat polyclonal (D20) antibodies and isotype controls were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse, goat, and rabbit anti-IgG antibody horseradish conjugates were from Vector Laboratories (Burlingame, CA). Rat anti-IgG antibody horseradish conjugate was from Jackson ImmunoResearch (West Grove, PA). The control siRNA and the siRNA for arrestin-2 and arrestin-3 were from the siGENOME SMARTpool commercially purchased from Dharmacon RNA Technologies (Lafayette, CO). The sequence of the siRNA that targets both human arrestin-2 (nucleotides 179–190 with respect to the start codon) and arrestin-3 (nucleotides 175–193 with respect to the A of the start codon) is 5′-ACCUGCGCCUUCCGCUAUG-3′ and was also from Dharmacon. Stromal cell-derived factor-1α (also called CXCL12) was from PeproTech (Rocky Hill, NJ).

Degradation Assay—Agonist-promoted degradation of HA-tagged CXCR4 in HEK293 cells and endogenous CXCR4 in HeLa cells was detected by immunoblot analysis, as previously described (7). Briefly, the cells were treated with vehicle (DMEM + 0.5% FBS) or CXCL12 (10 nM) in the presence of 50 μg/ml cycloheximide (to prevent protein synthesis) for 3 h. We have found that 10 nM CXCL12 produces the same level of CXCR4 degradation as 100 nM CXCL12 (20). The cells were directly lysed in 2× sample buffer, and receptor levels were detected as we have previously shown (6, 7, 20, 21). For RNA interference experiments, HeLa cells were transfected with control siRNA or siRNA directed against arrestin-2 (100 nM), arrestin-3 (100 nM), or arrestin-2 and arrestin-3 together (50 nM each) using Lipofectamine 2000, according to the manufacturer's instructions. For studies in HEK293 cells, the cells were transfected with siRNA as described for HeLa cells, followed by transfection with HA-CXCR4 the following day using FuGENE 6 transfection reagent, according to the manufacturer's instructions.

Detection of Ubiquitinated CXCR4—HEK293 cells grown on 10-cm dishes were transfected with arrestin-2, arrestin-3, arrestin-2/3, and control siRNA as described above, followed by co-transfection of HA-CXCR4 and FLAG-ubiquitin the next day. Detection of ubiquitinated CXCR4 was as previously described (7).

Measuring Receptor Internalization—HEK293 cells grown on 10-cm dishes were transfected with siRNA for arrestin-2 and control siRNA as described above, followed by co-transfection of HA-CXCR4 the next day. Receptor internalization was measured by cell surface enzyme-linked immunosorbent assay, as previously described (6).

Co-immunoprecipitation Studies—HEK293 cells stably expressing HA-tagged CXCR4 were serum-starved in DMEM supplemented with 0.5% FBS overnight (14–16 h) followed by treatment with the same medium without or with 30 nM CXCL12 for 15, 30, and 60 min. After stimulation, the cells were placed on ice, lysed in ice-cold immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, phosphatase inhibitor cocktails I and II (Sigma), and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A)). The lysates were clarified by centrifugation at 21,000 × *g* for 20 min at 4 °C, and equal amounts of clarified lysates were processed. An anti-AIP4 monoclonal antibody (G11; Santa Cruz) was used to immunoprecipitate endogenously expressed AIP4 in HEK293 cells, essentially as previously described (7), followed by SDS-PAGE and immunoblotting to detect endogenous arrestins.

GST Pulldowns—*Escherichia coli* BL21 cells transformed with GST fusion constructs were grown overnight at 37 °C. The following day, the cultures were diluted 1:50 and grown to an $A_{600} \sim 0.4–0.5$ at 37 °C and then induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 18 °C. The cells were pelleted, resuspended in binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin), and sonicated on ice. The lysates were clarified by centrifugation for 20 min at 21,000 × *g* at 4 °C followed by incubation with glutathione-Sepharose 4B beads overnight at 4 °C while rocking. The samples were washed and resuspended in binding buffer. For binding experiments using transfected cells, 1–3 μg of GST fusion proteins were incubated with cell lysates prepared from HEK293 cells expressing the protein of interest for 12–16 h. The samples were washed three times in binding buffer, eluted in 2× sample buffer by boiling for 10 min, and analyzed by SDS-PAGE and Western blotting. For binding experiments using purified proteins, 1 μg of GST fusion protein

was incubated with 300 ng of purified arrestin-2 for 1 h and processed in the same manner.

Immunofluorescence Microscopy—HEK293 cells were transfected with arrestin-2 siRNA followed by transfection of HA-CXCR4 tagged with yellow fluorescent protein at its carboxyl-terminal end (HA-CXCR4-YFP). The cells were treated with CXCL12 for 3 h, followed by fixation, permeabilization, and immunostaining for EEA1 and LAMP2. For AIP4 and arrestin-2 co-localization experiments, HEK293 cells transiently co-transfected with HA-CXCR4, mCh-AIP4, and YFP-arrestin-2 were serum-starved for 14–16 h in DMEM containing 0.5% FBS and treated with vehicle or 30 nM CXCL12 for 30 min followed by permeabilization and fixation. The images were acquired using a Zeiss LSM 510 laser scanning confocal imaging system with a 63× W Apochromat objective at 512 × 512 resolution. The acquired images were analyzed using Image J 1.3v software (NIH).

Statistical Analysis—The data were analyzed and subjected to statistical analyses using Prism 4.0 software (GraphPad Software, Inc.).

RESULTS

To elucidate the mechanisms mediating CXCR4 degradation and ubiquitination, we initially examined the effect of siRNA-mediated depletion of arrestin-2 and arrestin-3 on agonist promoted degradation of endogenous CXCR4 in HeLa cells. As shown in Fig. 1 (*A* and *B*), agonist-promoted degradation of CXCR4 was inhibited in cells transfected with either arrestin-2 or arrestin-3 siRNA or both, suggesting that arrestins are involved in agonist-promoted degradation of CXCR4. Interestingly, inhibition of CXCR4 degradation was more pronounced in cells depleted of arrestin-2 compared with arrestin-3, even though the siRNA-mediated depletion of arrestin-3 was consistently greater than depletion of arrestin-2, suggesting a more important role for arrestin-2 in degradation of activated CXCR4.

We next examined the role of arrestin-2 in agonist-promoted CXCR4 degradation in HEK293 cells, a model cell line used previously to study CXCR4 trafficking (6, 7). We found that degradation of HA-tagged CXCR4 induced by agonist was significantly inhibited in arrestin-2-depleted cells as compared with control siRNA transfected cells (Fig. 1, *C* and *D*), consistent with that observed with endogenous CXCR4 in HeLa cells. We have previously shown that internalization is a prerequisite for degradation (6), and because arrestins are thought to play a role in CXCR4 internalization (15, 17), we next determined whether inhibition of internalization could account for the attenuated degradation observed in the arrestin-2 siRNA-treated cells. HEK293 cells were transfected with siRNA targeting arrestin-2 or control siRNA together with HA-tagged CXCR4, and receptor internalization was assessed by cell surface enzyme-linked immunosorbent assay, as previously described (6). As shown in Fig. 1*E*, agonist-promoted internalization of CXCR4 in arrestin-2-depleted cells was similar to that in control siRNA-transfected cells, suggesting that arrestin-2 is not involved in agonist-promoted internalization of CXCR4.

Direct ubiquitination of CXCR4 at the plasma membrane is required for its sorting to the degradative pathway but not for its internalization (6, 7). Because arrestin-2 also appears to be required for CXCR4 degradation but not internalization, we next assessed whether the effect on CXCR4 degradation is due to a role on CXCR4 ubiquitination. HEK293 cells transfected with siRNA targeted against arrestin-2 and arrestin-3 or control siRNA plus HA-tagged CXCR4 and FLAG-tagged ubiquitin were stimulated with or without agonist for 30 min, followed by receptor immunoprecipitation and immunoblotting to detect the incorporation of tagged ubiquitin, as previously described (7). As shown in Fig. 1*F*, ubiquitination of CXCR4 was not affected in arrestin-2 or arrestin-3 siRNA depleted cells as compared with control siRNA-treated cells, even though arrestins were substantially depleted (Fig. 1*F*, *bottom panel*). In addition, the ubiquitination status of CXCR4 was not altered in cells that were transfected with a siRNA that targets both arrestin-2 and arrestin-3 with respect to mock transfected cells (Fig. 1*G*), further suggesting that arrestins are not involved in CXCR4 ubiquitination. Taken together, these data indicate that arrestin-2 is not involved in CXCR4 ubiquitination and internalization and suggest that its role in CXCR4 degradation is likely due to a post-internalization function such as endosomal sorting into the degradative pathway.

We next examined the distribution of CXCR4 in arrestin-2-depleted HEK293 cells that were treated with or without CXCL12 using confocal immunofluorescence microscopy. In the absence of stimulation, CXCR4 was localized predominantly to the plasma membrane in both control and arrestin-2 siRNA-treated cells, although a portion of CXCR4 was also localized to intracellular puncta that partially overlapped with the early endosomal marker EEA1 (Fig. 2*A*, *top panels*, control and arrestin-2) or late endosomal/lysosomal marker LAMP2 (Fig. 2*B*, *top panels*, control and arrestin-2), suggesting that CXCR4 undergoes constitutive internalization and trafficking to early endosomes and lysosomes. Upon treatment with CXCL12 for 3 h, in the control treated cells, CXCR4 mostly co-localized with the late endosomal/lysosomal marker LAMP2 (Fig. 2*B*), but not with the early endosomal marker EEA1 (Fig. 2*A*), suggesting that CXCR4 traffics efficiently to lysosomes upon stimulation with CXCL12. In contrast, upon stimulation with CXCL12 in the arrestin-2 siRNA-treated cells, CXCR4 mostly co-localized with EEA1 and not LAMP2, suggesting that transit from endosomes to lysosomes is impaired as compared with control treated cells. Taken together, these data suggest that arrestin-2 is required for proper CXCR4 trafficking from early endosomes to lysosomes.

We previously showed that AIP4 is localized to endosomes and mediates endosomal sorting of CXCR4 (7); therefore, we next asked whether arrestins facilitate AIP4-mediated trafficking of CXCR4. To address this, we initially determined whether arrestins interact with AIP4 in cells. HEK293 cells were transiently transfected with HA-tagged arrestin-2 or arrestin-3 and FLAG-tagged AIP4 or empty vector (pCMV). Whole cell lysates were subject to immunoprecipitation using a FLAG antibody followed by immunoblotting to detect arrestin-2 or arrestin-3 in the immunoprecipitates. As shown in Fig. 3*A*, arrestin-2 and arrestin-3 co-immunoprecipitation was observed in cells transfected with FLAG-tagged AIP4 but not in

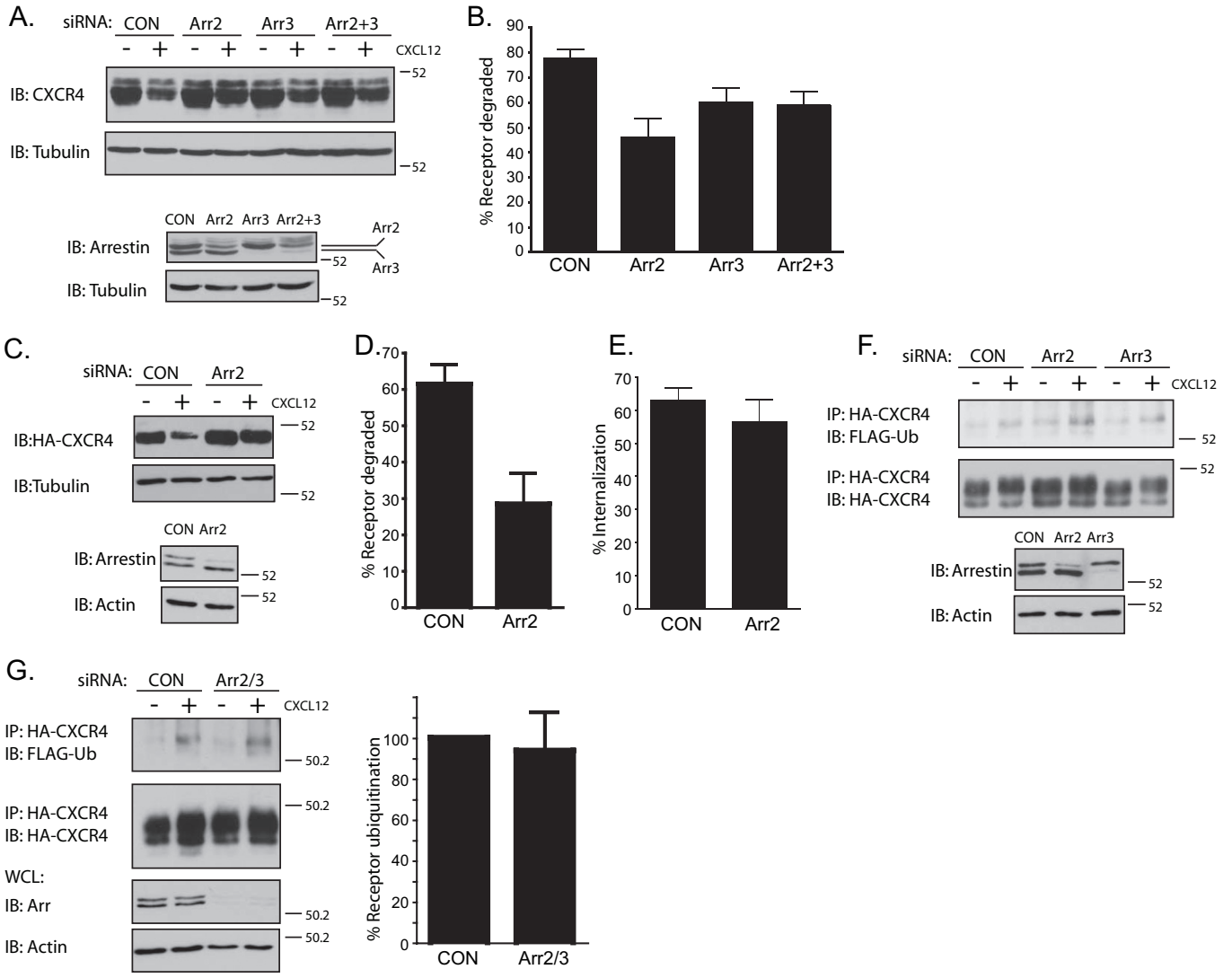

FIGURE 1. **Arrestin depletion inhibits degradation but not internalization and ubiquitination of CXCR4.** *A*, arrestin siRNA attenuates agonist-mediated degradation of endogenous CXCR4 in HeLa cells. HeLa cells were transfected with control siRNA (*CON*) or siRNA against arrestin-2 (*Arr2*; final concentration, 100 nM), arrestin-3 (*Arr3*; final concentration, 100 nM), or arrestin-2 and arrestin-3 together (*Arr2+3*; final concentration for each, 50 nM). The cells were treated with vehicle (DMEM containing 0.5% FBS) or CXCL12 (10 nM) for 3 h in DMEM containing 10% FBS supplemented with 50 μg/ml cycloheximide. Equal amounts of cell lysates were subject to SDS-PAGE followed by immunoblotting for endogenous receptor levels. The arrestin levels were assessed using an antibody that recognizes both arrestin-2 and arrestin-3 from the indicated siRNA transfected cells. The immunoblots were stripped and reprobed for tubulin to assess loading. Shown are representative immunoblots (*IB*). The molecular weights are indicated. *B*, graphical representation of CXCR4 degradation. Immunoblots were subject to densitometric analysis to quantify receptor levels. The *bar graphs* represent the average percentages of receptor degraded in cells treated with CXCL12 as compared with vehicle-treated cells after normalization to tubulin levels. The *error bars* represent the standard errors of the mean from six independent experiments. The data were subjected to statistical analysis by a repeated measures analysis of variance followed by a post-hoc Dunnett's multiple comparison test (p value <0.01). *C*, arrestin-2 siRNA attenuates agonist-mediated degradation of HA-CXCR4 in HEK293 cells. HEK293 cells were transfected with control siRNA (*CON*) or siRNA against arrestin-2 (*Arr2*) followed by transfection with HA-tagged CXCR4. After 72 h, the cells were treated with vehicle (DMEM containing 0.5% FBS) or CXCL12 (10 nM) for 3 h in DMEM containing 10% FBS supplemented with 50 μg/ml cycloheximide. Equal amounts of cell lysates were subjected to SDS-PAGE followed by immunoblotting to detect HA-CXCR4. The arrestin levels were assessed using an antibody that recognizes both arrestin-2 and arrestin-3 from the indicated siRNA-transfected cells. The immunoblots were stripped and reprobed for tubulin or actin to assess loading. Shown are representative immunoblots. The molecular weights are indicated. *D*, graphical representation of HA-CXCR4 degradation. The immunoblots were subjected to densitometric analysis to quantify receptor levels. The *bar graphs* represent the average percentages of receptor degraded in cells treated with CXCL12 as compared with vehicle-treated cells after normalization to tubulin levels. The *error bars* represent the standard errors of the mean from four independent experiments. To determine the statistical significance, the data were subjected to an unpaired *t* test (p value <0.01). *E*, arrestin-2 does not mediate agonist-induced internalization of HA-CXCR4. HEK293 cells were transfected with control siRNA (*CON*) or siRNA against arrestin-2 (*Arr-2*) followed by transfection with HA-tagged CXCR4. The cells were treated with 30 nM CXCL12 for 20 min and CXCR4 internalization was assessed by cell surface enzyme-linked immunosorbent assay. The *bar graphs* represent the percentages of average cell surface receptor levels internalized in cells treated with CXCL12 as compared with vehicle treated cells. The *error bars* represent the standard errors of the mean from four independent experiments. *F* and *G*, depletion of arrestin-2 and arrestin-3 does not affect agonist-induced CXCR4 ubiquitination. HEK293 cells were transfected with control siRNA (*CON*) or siRNA targeting arrestin-2 (*Arr2*) or arrestin-3 (*Arr3*) (*F*). The cells were mock (*CON*) transfected or transfected with siRNA that recognizes both arrestin-2 and arrestin-3 (Arr2/3) (*G*), followed by transfection with DNA encoding HA-CXCR4 and FLAG-ubiquitin. The cells were stimulated with 100 nM CXCL12 for 30 min, and CXCR4 ubiquitination was determined as previously described (6, 7). The arrestin levels were assessed using an antibody that recognizes both arrestin-2 and arrestin-3 from the indicated siRNA transfected cells. The immunoblots were stripped and reprobed for actin to assess loading. Shown are representative immunoblots. The molecular weights are indicated. The ubiquitinated CXCR4 levels were assessed by densitometric analysis and the fold increase upon CXCL12 treatment as compared with vehicle treatment was normalized to receptor levels present in the immunoprecipitates (*IP*). The *bar graph* represents receptor ubiquitination observed in arrestin-2/3 siRNA (*Arr2/3*)-treated cells as compared with control (*CON*) treated cells. The *error bars* represent the S.E. from three independent experiments.

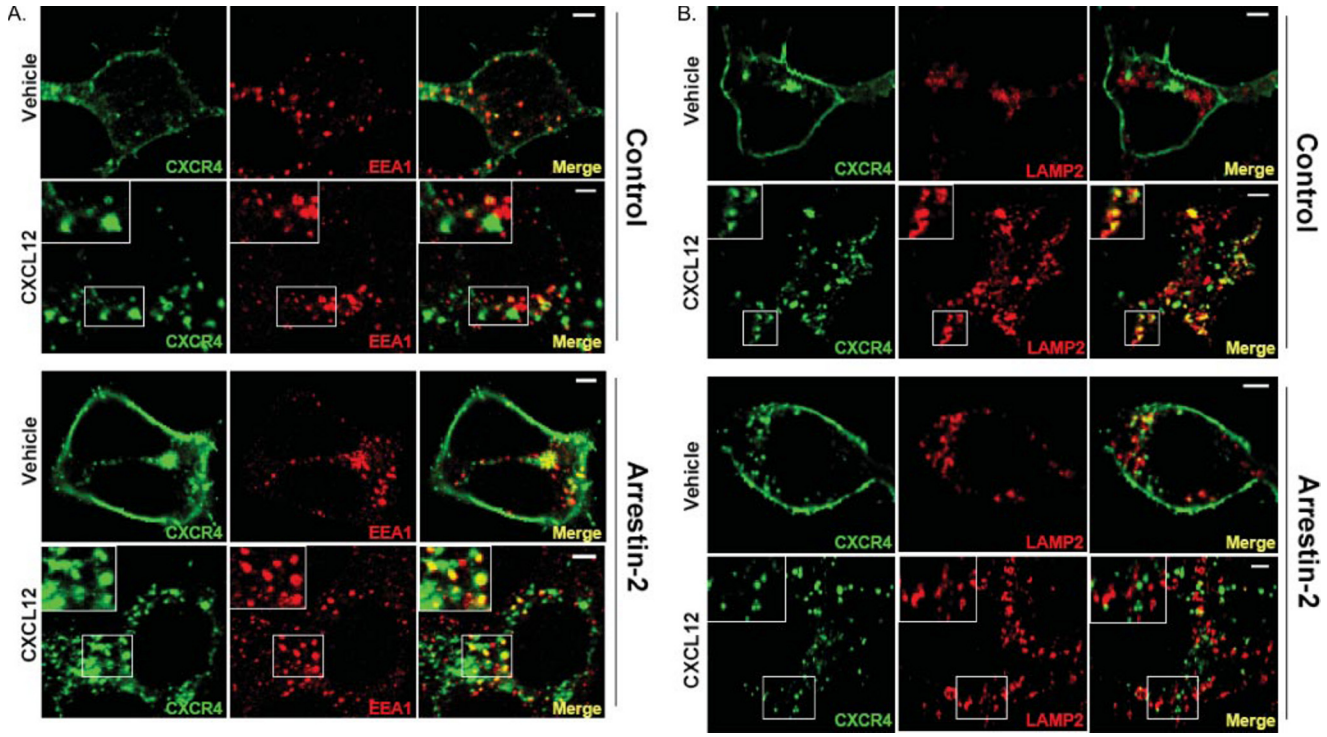

FIGURE 2. **CXCR4 accumulates on early endosomes and does not traffic to lysosomes in arrestin-2 depleted cells.** HEK293 cells were transfected with arrestin-2 siRNA followed by transfection of HA-CXCR4-YFP. The cells were stimulated with 10 nM CXCL12 for 3 h and then fixed, permeabilized, and immunostained for either EEA1 (*A*), an early endosomal marker, or LAMP-2 (*B*), a late endosomal/lysosomal marker. CXCR4 is shown *green* (*left panels*), and EEA1 (*A*) and LAMP-2 (*B*) are shown *red* (*middle panels*). Puncta that appear *yellow* in the merged images (*right panels*) indicate co-localization of CXCR4 with the endosomal markers. The *insets* represent enlarged views of the boxed regions. The *bar* represents 5 microns. Similar results were observed from three independent experiments.

vector-transfected cells, suggesting that arrestins associate with AIP4 in cells.

To define the nature of the interaction, we examined whether AIP4 and arrestin-2 interact directly or via an intermediate protein. Purified arrestin-2 was incubated with glutathione beads that were coated with full-length AIP4 fused to GST or GST alone (shown schematically in Fig. 3*B*), and bound arrestin-2 was detected by immunoblotting. As shown in Fig. 3*C*, GST-AIP4, but not GST alone, bound to purified arrestin-2, suggesting a direct interaction between AIP4 and arrestin-2. AIP4 generally binds to its target proteins via its four tandem WW domains (19, 22); therefore, we next asked whether the interaction with arrestin-2 is mediated by the WW domains. As shown in Fig. 3*C*, arrestin-2 also bound to a GST fusion protein of the AIP4 WW domains (GST-WW-I-IV); however, GST-AIP4 appeared to bind better than GST-WW-I-IV, suggesting that there may be additional sites on AIP4 that bind to arrestin-2. Alternatively, full-length AIP4 may maintain a more favorable arrestin-2 binding conformation. Similarly, we observed better binding of GST-AIP4 than GST-WW-I-IV to HA-tagged arrestin-2 expressed in HEK293 cells (Fig. 3*D*). Thus, it appears that the WW domains, at least in part, mediate the interaction with arrestin-2. To explore which of the four WW domains of AIP4 mediated the interaction with arrestin-2, we examined the ability of each individual WW domain to bind to arrestin-2. Equal amounts of GST fusion proteins containing individual WW domains were assessed for their ability to interact with HA-tagged arrestin-2 in whole cell lysates. As shown in Fig. 3*E*, the interaction with arrestin-2 is mediated by WW domains I and II, but not WW domains III and IV of AIP4.

WW domains interact with proline-rich motifs in various contexts, and because arrestins contain several proline-rich sequences, we decided to use truncation mutagenesis to determine the AIP4-binding regions on arrestin-2. Various arrestin-2 truncation mutants used in these studies are shown schematically in Fig. 4*A*. Initially, we examined two truncation mutants encompassing the amino-terminal (amino acids 1–260) and carboxyl-terminal (amino acids 261–418) regions of arrestin-2. Whole cell lysates of HEK293 cells expressing HA-tagged arrestin-2 truncation mutants were incubated with glutathione beads coated with WW domains I–IV fused to GST or GST alone. As shown in Fig. 4*B*, GST-WW-I-IV bound to arrestin-2-(1–260) but not arrestin-2-(261–418). Further truncation of arrestin-2-(1–260) to residues 179 and 161 revealed that binding to AIP4 was most likely mediated by residues located between residues 161 and 260. Interestingly, this region contains a proline-rich stretch within the hinge region (174–181) that connects the amino- and carboxyl-terminal domains of arrestin-2 (23). Mutation of two proline residues to alanines (arrestin-2-(P180A/P182A)) and deletion of 8 amino acids (Δ175–182) encompassing the proline residues within this region in the context of the 1–260 construct failed to abrogate binding to GST-WW-I-IV (data not shown), suggesting that these residues are not important for binding to AIP4 and that other regions may be involved.

To gain further insight into the mechanism by which arrestins function in AIP4-dependent degradation of CXCR4,

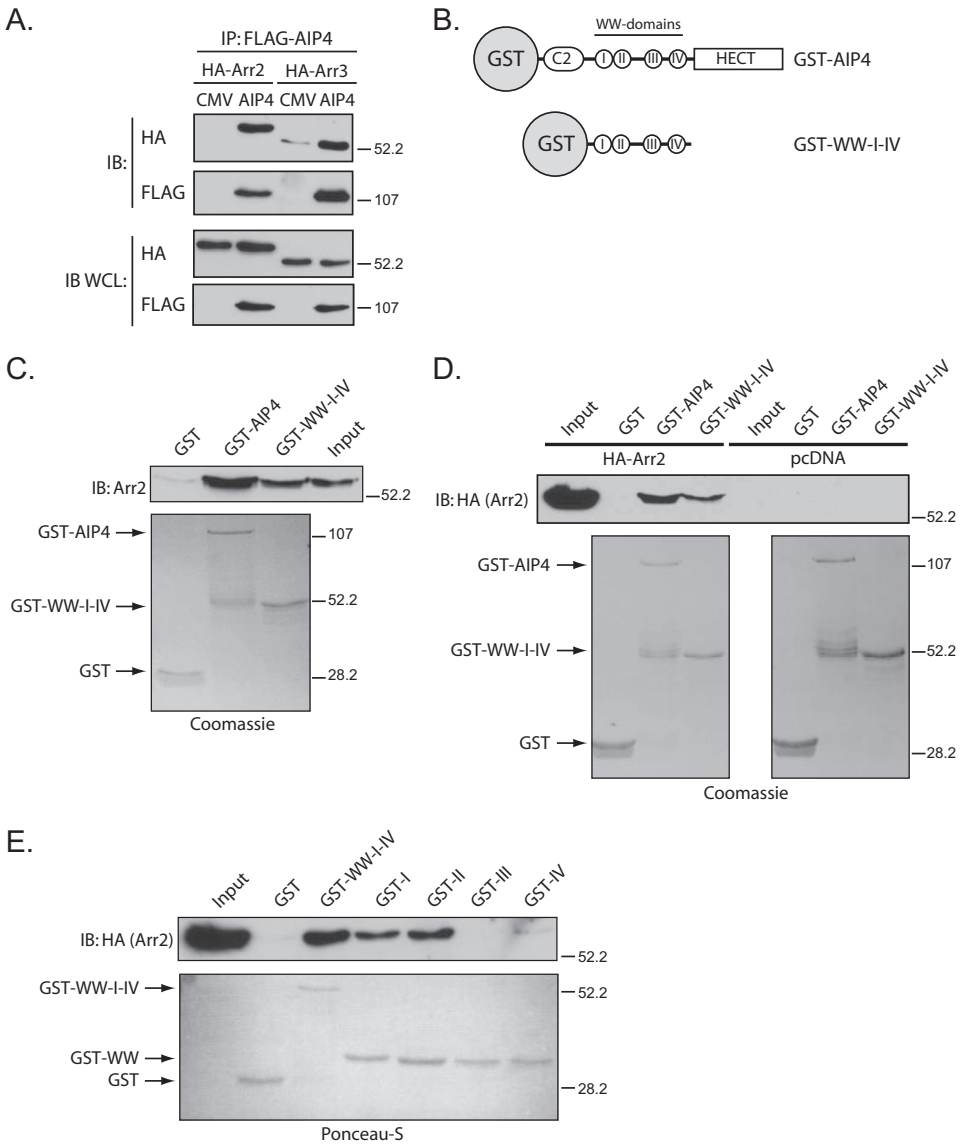

FIGURE 3. **Arrestin-2-binding domains on AIP4.** *A*, AIP4 interacts with nonvisual arrestins. HEK293 cells were transiently co-transfected with FLAG-tagged full-length AIP4 or empty vector (pCMV) and HA-tagged arrestin-2 or arrestin-3. Whole cell lysates (WCL) were subject to immunoprecipitation (*IP*) using an anti-FLAG M2 monoclonal antibody followed by SDS-PAGE and immunoblotting to detect HA-tagged arrestins in the immunoprecipitates. Approximately 1% of WCL used in the immunoprecipitation reaction and one-third of the immunoprecipitation sample were immunoblotted (*IB*) to determine the levels of each protein. The molecular weights are indicated. Shown are representative blots from four independent experiments. *B*, schematic representation of AIP4 GST fusion constructs. AIP4 is characterized by the presence of a C2 domain, four tandem WW domains, and a catalytic homologous to E6-AP carboxyl-terminal domain. Full-length AIP4 (GST-AIP4) and a construct encoding the four WW domains (GST-WW-I-IV) were cloned into pGEX-4T2. *C*, AIP4 interacts directly with arrestin-2. The indicated GST fusion proteins (≈1 μg) were incubated with purified arrestin-2 (300 ng) followed by SDS-PAGE and immunoblotting with an anti-arrestin antibody to detect arrestin-2. The input represents 10 ng of purified arrestin-2. A Coomassie-stained gel is shown to indicate the levels of the GST fusion proteins used in the binding experiment. The extra bands in the GST-AIP4 lanes represent degradation products co-purified with full-length GST-AIP4. Shown are representative blots from three independent experiments. The molecular weights are indicated. *D*, the WW domains of AIP4 mediate the interaction with arrestin-2. WCL from HEK293 cells expressing HA-arrestin-2 or pcDNA were incubated with GST-AIP4, GST-WW-I-IV, or GST alone followed by SDS-PAGE and immunoblotting to detect HA-arrestin-2 binding. The input equals 2% of the lysate that was used in the binding experiment. A Coomassie-stained gel is shown to indicate the levels of the GST fusion proteins used in the binding experiment. The extra bands in the GST-AIP4 lanes represent degradation products co-purified with full-length GST-AIP4. Shown are representative blots from three independent experiments. The molecular weights are indicated. *E*, WW domains I and II interact with arrestin-2. WCL from HEK293 cells expressing HA-arrestin-2 were incubated with GST-AIP4, GST-WW-I-IV, GST fused to each of the individual four WW domains, or GST alone followed by SDS-PAGE and immunoblotting to detect HA-arrestin-2 binding. The input equals 0.5% of the lysate that was used in the binding experiment and approximately one-half of bound sample was immunoblotted. The *upper blot* was stained with Ponceau S to show the levels of the GST fusion proteins used in the binding experiment. Shown are representative blots from three independent experiments. The molecular weights are indicated.

we examined whether receptor activation promotes the interaction between AIP4 and arrestin-2. Initially, we compared the ability of AIP4 to bind to wild-type arrestin-2 and arrestin-2-R169E. The arrestin-2-R169E mutant is thought to mimic the form of arrestin when it is bound to activated and phosphorylated GPCRs and is thus considered to be "constitutively active" (24). HEK293 cells were transiently transfected with either arrestin-2 wild-type or R169E mutant together with FLAG-tagged AIP4 or empty vector (pCMV). Whole cell lysates were subject to immunoprecipitation using the FLAG antibody followed by immunoblotting to detect arrestin-2 in the immunoprecipitates. As shown in Fig. 5*A*, a single band corresponding to arrestin-2 was observed in cells transfected with FLAG-tagged AIP4 but not in cells transfected with empty vector. Interestingly, arrestin-2-R169E showed enhanced binding to FLAG-tagged AIP4 as compared with wild-type arrestin-2 (Fig. 5*A*), suggesting that receptor activation may promote arrestin-2 binding to AIP4.

To confirm our findings, we next examined whether activation of CXCR4 promotes the association between endogenous AIP4 and arrestin-2 in HEK293 cells that stably express HA-tagged CXCR4. The cells were treated in the absence or presence of CXCL12 followed by immunoprecipitation of endogenous AIP4 and immunoblotting to detect arrestins in the immunoprecipitates. As shown in Fig. 5*B*, incubation of cells with agonist for 15–30 min induced a robust increase in the amount of arrestin-2 and arrestin-3 detected in AIP4 immunoprecipitates, which diminished by 60 min, indicating that activation of CXCR4 promotes a time-dependent interaction between AIP4 and arrestins.

To determine whether AIP4 and arrestin-2 co-localize within the same subcellular compartment, we employed immunofluorescence confocal microscopy on HEK293 cells co-expressing HA-CXCR4,

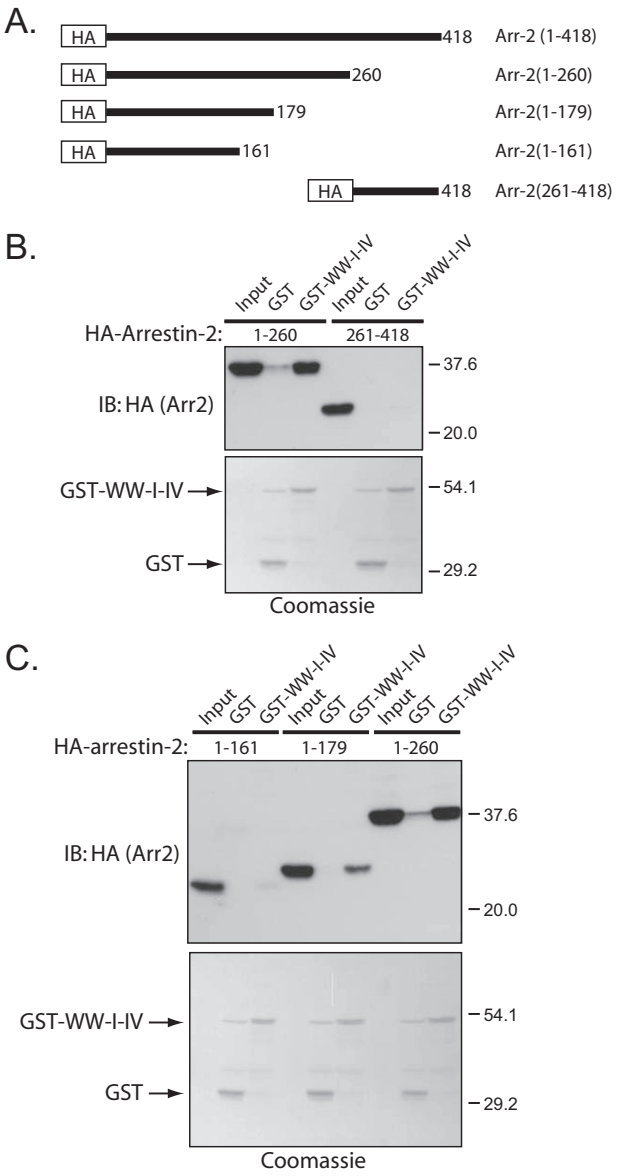

FIGURE 4. **AIP4-binding domains on arrestin-2.** *A*, schematic representation of arrestin-2 constructs. Truncated versions of arrestin-2 were generated by PCR and inserted into pcDNA3 in-frame with HA-tag at the amino-terminal end. *B* and *C*, AIP4-binding site is located on the amino-terminal half of arrestin-2. WCL from HEK293 cells transiently expressing HA-tagged truncated versions of arrestin-2, 1–260 and 261–418 (*B*) and 1–260, 1–179 and 1–161 (*C*), were incubated with glutathione beads coupled to GST-WW-I-IV and GST alone. The bound samples were resolved by SDS-PAGE, followed by immunoblotting (*IB*) with an anti-HA-antibody (*top panel*) and Coomassie staining (*bottom panel*). The input represents ~0.5% of the lysate that was used in the binding experiment, and one-third of the bound sample was immunoblotted. The extra bands in the Coomassie-stained gel represent proteins in the WCL that bound nonspecifically to GST during the binding experiment. Shown are representative blots from three independent experiments. The molecular weights are indicated.

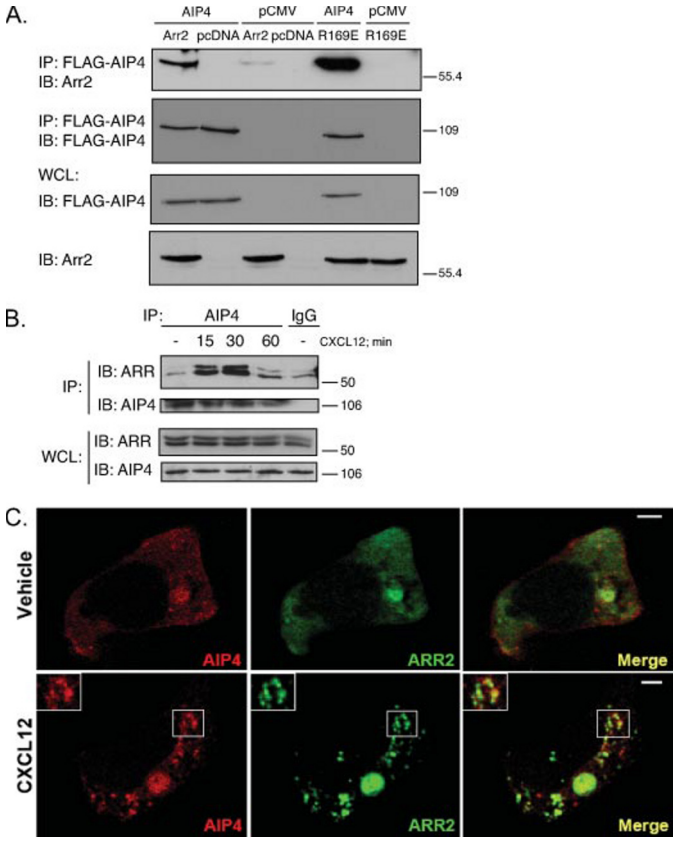

FIGURE 5. **AIP4 interacts with arrestin in an activation-dependent manner.** *A*, AIP4 shows enhanced binding to the constitutively active arrestin-2-R169E mutant. HEK293 cells were transiently transfected with cDNA encoding arrestin-2, arrestin-2-R169E, FLAG-tagged full-length AIP4, pcDNA3, and pCMV. WCL were subject to immunoprecipitation (*IP*) using the anti-FLAG M2 monoclonal antibody and immunoblotting using an arrestin polyclonal antibody. Shown are representative blots from four independent experiments. WCL were immunoblotted (*IB*) to detect expression of each construct. The molecular weights are indicated. *B*, the interaction between AIP4 and arrestin-2 is mediated by activation of CXCR4. HEK293 cells stably expressing HA-tagged CXCR4 were serum-starved for 14–18 h and treated with vehicle or CXCL12 (30 nM) for 15–60 min. Equal amounts of WCL were subject to immunoprecipitation with an anti-AIP4 monoclonal antibody (G11) or an isotype control antibody (mouse IgG_1) and IB as indicated. Shown are representative blots from three independent experiments. The molecular weights are indicated. *C*, arrestin-2 co-localizes with AIP4 on endosomes. HEK293 cells transiently co-transfected with HA-CXCR4, mCh-AIP4, and YFP-arrestin-2 were serum-starved for 14–18 h and incubated in the presence of 30 nM CXCL12 or vehicle for 30 min. The cells were permeabilized, fixed, and analyzed by confocal microscopy. AIP4 is shown in *red* (*left panels*), whereas arrestin-2 (*middle panels*) is shown *green*. Puncta that co-localize appear *yellow* in the merged images (*right panels*). The *insets* represent enlarged views of *boxed regions*. *Scale bar*, 5 μm. Similar results were observed from three independent experiments.

YFP-arrestin-2, and mCh-AIP4. The cells were treated with agonist for 30 min, permeabilized, fixed, and observed using a confocal microscope. As shown in Fig. 5*C*, in the vehicle-treated cells, arrestin-2 and AIP4 were mostly diffuse, although AIP4 was also found in puncta, consistent with its localization to endosomes (7). In contrast, in the CXCL12-treated cells, arrestin-2 became punctate and showed strong co-localization with AIP4. Taken together, these data suggest that arrestin-2 co-localizes with AIP4 on endosomes upon CXCR4 activation, which may facilitate its ability to mediate sorting of CXCR4 into the degradative pathway.

DISCUSSION

Here, we establish that arrestin-2 functions in endosomal sorting of CXCR4 into the degradative pathway (7). Arrestin-2 likely facilitates AIP4-mediated events critical for endosomal sorting of CXCR4, although the precise mechanism is not known. Arrestin-2 is likely not a target for ubiquitination by AIP4 because siRNA-mediated depletion of AIP4 did not have any effect on the basal ubiquitination status of arrestin-2 (data

not shown). Arrestin-2 might facilitate CXCR4 stimulated ubiquitination of HRS, which is AIP4-dependent (7). Interestingly, HRS also mediates lysosomal targeting of the epidermal growth factor receptor. However, AIP4, despite its co-localization with HRS on endosomes, functions in sorting CXCR4 and not epidermal growth factor receptor, suggesting that the endosomal sorting activity of AIP4 is specific for a subset of receptors targeted to lysosomes (7, 21). It is possible that the specificity of AIP4 for CXCR4 endosomal sorting is a function of its ability to bind to arrestin-2 on endosomes. Regardless, our data support a model whereby arrestin-2 interacts with AIP4 on endosomes, where it likely modulates endosomal functions of AIP4 that are critical for lysosomal targeting of CXCR4 (7, 21).

Although the interaction between AIP4 and arrestin-2 in cells is regulated by activation of CXCR4, we can detect basal binding between the proteins when overexpressed in the absence of activated receptor (Fig. 3). It may be possible that the AIP4-binding site is only partially accessible in the inactive conformation and that arrestin-2 activation makes it more accessible leading to greater AIP4 binding. Alternatively, it is possible that there are multiple AIP4-binding sites in arrestin-2. For example, a low affinity binding site may be accessible when arrestin-2 is in the inactive conformation, and a second, high affinity site may become accessible upon arrestin-2 activation, leading to a stronger interaction with AIP4. Nevertheless, our data indicate that AIP4 and arrestin-2 interact directly *in vitro*, and their association in cells is regulated by CXCR4 activation. In addition, we have identified the amino-terminal region of arrestin-2 and AIP4 WW domains I and II as the binding regions that may facilitate the interaction in cells.

Typically, arrestin binding to activated and phosphorylated GPCRs occurs at the plasma membrane, which is thought to promote a conformational change in arrestin exposing the buried carboxyl-terminal end that binds to partners such as clathrin and β-adaptin (13). Because CXCR4 activation promotes AIP4 binding to arrestin-2, arrestin-2 may be recruited to phosphorylated CXCR4 before it interacts with AIP4. However, because the AIP4-binding site maps to the amino-terminal half of arrestin-2, we believe the mechanism of the interaction will be distinct from that for clathrin and β-adaptin, although a conformational change in arrestin-2 is likely required.

Interestingly, the arrestin-2-AIP4 interaction is maximal after 15–30 min of agonist treatment, when both proteins co-localize on endocytic vesicles. Thus, it is possible that arrestin-2 binds to phosphorylated CXCR4 and AIP4 at the plasma membrane, and all three proteins co-traffic as a complex to endosomes. Arrestins have been shown to form stable complexes with certain GPCRs and several signaling molecules that persist for some time on endosomes (25), but whether this is true for CXCR4, AIP4, and arrestin-2 remains to be determined. It is also possible that arrestin-2 interacts with phosphorylated CXCR4 at the plasma membrane, which co-internalize as a complex and once on endosomes somehow interacts with the endosomal pool of AIP4. This may also explain why arrestin-2 is not important for ubiquitination of the receptor at the plasma membrane. We show that in addition to not being involved in the ubiquitination of CXCR4, arrestin-2 is not involved in its internalization. It is possible that arrestin-2 mediates desensitization of CXCR4, although this function has been previously assigned to arrestin-3 (16), which possibly also mediates CXCR4 internalization (15, 17). Alternatively, arrestin-2 may instead be directly recruited to endosomes by activated CXCR4, where it carries out its endosomal sorting function. Regardless of the mechanism, our data reveal that arrestin-2 co-localizes with AIP4 on endocytic vesicles, where it likely functions to mediate lysosomal targeting of CXCR4.

In conclusion, we describe a novel function for arrestin-2 as an endosomal sorting molecule together with AIP4 to mediate CXCR4 entry into a degradative pathway. This function deviates from the general paradigm that arrestins serve as adaptors for clathrin-dependent internalization of GPCRs at the plasma membrane and suggests that arrestins may also serve as endosomal adaptor proteins required for sorting GPCRs into the degradative pathway.

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REFERENCES

1. Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T. (1999) *Science* **283,** 845–848
2. Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. (1998) *Nature* **393,** 591–594
3. Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998) *Nature* **393,** 595–599
4. Diaz, G. A., and Gulino, A. V. (2005) *Curr. Allergy Asthma Rep.* **5,** 350–355
5. Kakinuma, T., and Hwang, S. T. (2006) *J. Leukocyte Biol.* **79,** 639–651
6. Marchese, A., and Benovic, J. L. (2001) *J. Biol. Chem.* **276,** 45509–45512
7. Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (2003) *Dev. Cell* **5,** 709–722
8. Angers, A., Ramjaun, A. R., and McPherson, P. S. (2004) *J. Biol. Chem.* **279,** 11471–11479
9. Ingham, R. J., Gish, G., and Pawson, T. (2004) *Oncogene* **23,** 1972–1984
10. Slagsvold, T., Pattni, K., Malerod, L., and Stenmark, H. (2006) *Trends Cell Biol.* **16,** 317–326
11. Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (2002) *Nat. Cell Biol.* **4,** 394–398
12. Sachse, M., Urbe, S., Oorschot, V., Strous, G. J., and Klumperman, J. (2002) *Mol. Biol. Cell* **13,** 1313–1328
13. Marchese, A., Chen, C., Kim, Y. M., and Benovic, J. L. (2003) *Trends Biochem. Sci.* **28,** 369–376
14. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294,** 1307–1313
15. Cheng, Z. J., Zhao, J., Sun, Y., Hu, W., Wu, Y. L., Cen, B., Wu, G. X., and Pei, G. (2000) *J. Biol. Chem.* **275,** 2479–2485
16. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) *Proc. Natl. Acad Sci. U. S. A.* **99,** 7478–7483
17. Orsini, M. J., Parent, J. L., Mundell, S. J., Marchese, A., and Benovic, J. L. (1999) *J. Biol. Chem.* **274,** 31076–31086
18. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) *Nat. Biotech.* **22,** 1567–1572

19. Winberg, G., Matskova, L., Chen, F., Plant, P., Rotin, D., Gish, G., Ingham, R., Ernberg, I., and Pawson, T. (2000) *Mol. Cell. Biol.* **20,** 8526–8535
20. Marchese, A., and Benovic, J. L. (2004) *Methods Mol. Biol.* **259,** 299–305
21. Slagsvold, T., Marchese, A., Brech, A., and Stenmark, H. (2006) *EMBO J.* **25,** 3738–3749
22. Ingham, R. J., Colwill, K., Howard, C., Dettwiler, S., Lim, C. S., Yu, J., Hersi, K., Raaijmakers, J., Gish, G., Mbamalu, G., Taylor, L., Yeung, B., Vassilovski, G., Amin, M., Chen, F., Matskova, L., Winberg, G., Ernberg, I., Linding, R., O'Donnell, P., Starostine, A., Keller, W., Metalnikov, P., Stark, C., and Pawson, T. (2005) *Mol. Cell. Biol.* **25,** 7092–7106
23. Milano, S. K., Pace, H. C., Kim, Y. M., Brenner, C., and Benovic, J. L. (2002) *Biochemistry* **41,** 3321–3328
24. Gurevich, V. V., and Gurevich, E. V. (2006) *Pharmacol. Ther.* **110,** 465–502
25. Shenoy, S. K., and Lefkowitz, R. J. (2005) *J. Biol. Chem.* **280,** 15315–15324