Novel Roles for the E3 Ubiquitin Ligase Atrophin-interacting Protein 4 and Signal Transduction Adaptor Molecule 1 in G Protein-coupled Receptor Signaling

Rohit Malik, Unice J. K. Soh, JoAnn Trejo, and Adriano Marchese

From the Department of Molecular Pharmacology and Therapeutics and the Program in Molecular Biology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153 and the Department of Pharmacology, University of California, San Diego, La Jolla, California 92093

Background: CXCR4 endosomal trafficking is regulated by the E3 ubiquitin ligase AIP4 and the ESCRT protein STAM-1.

Results: A discrete subpopulation of AIP4 and STAM-1 localized in caveolae form a complex and regulate CXCR4-induced ERK-1/2 activation.

Conclusion: AIP4 and STAM-1 serve distinct functions in regulation of CXCR4 endosomal sorting and signaling.

Significance: AIP4 and STAM-1 diversify the repertoire of GPCR signaling regulators.

The CXCL12/CXCR4 signaling axis plays an important role in human health and disease; however, the molecular mechanisms mediating CXCR4 signaling remain poorly understood. Ubiquitin modification of CXCR4 by the E3 ubiquitin ligase AIP4 is required for lysosomal sorting and degradation, which is mediated by the endosomal sorting complex required for transport (ESCRT) machinery. CXCR4 sorting is regulated by an interaction between endosomal localized arrestin-2 and STAM-1, an ESCRT-0 component. Here, we report a novel role for AIP4 and STAM-1 in regulation of CXCR4 signaling that is distinct from their function in CXCR4 trafficking. Depletion of AIP4 and STAM-1 by siRNA caused significant inhibition of CXCR4-induced ERK-1/2 activation, whereas overexpression of these proteins enhanced CXCR4 signaling. We further show that AIP4 and STAM-1 physically interact and that the proline-rich region in AIP4 and the SH3 domain in STAM-1 are essential for the interaction. Overexpression of an AIP4 catalytically inactive mutant and a mutant that shows poor binding to STAM-1 fails to enhance CXCR4-induced ERK-1/2 signaling, as compared with wild-type AIP4, suggesting that the interaction between AIP4 and STAM-1 and the ligase activity of AIP4 are essential for ERK-1/2 activation. Remarkably, a discrete subpopulation of AIP4 and STAM-1 resides in caveolar microdomains with CXCR4 and appears to mediate ERK-1/2 signaling. We propose that AIP4-mediated ubiquitination of STAM-1 in caveolae coordinates activation of ERK-1/2 signaling. Thus, our study reveals a novel function for ubiquitin in the regulation of CXCR4 signaling, which may be broadly applicable to other G protein-coupled receptors.

The CXCR4/CXCL12 (CXCL12 is also known as stromal cell-derived factor 1α) receptor/chemokine signaling axis has critical functions in development of the heart, brain, and vasculature and in tissue repair mechanisms (1–4). CXCR4-mediated signaling has also been linked to multiple pathological conditions including WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome and cancer progression (5, 6). Upon activation, CXCR4 couples to the heterotrimeric G protein, which leads to inhibition of adenylyl cyclase and to the mobilization of intracellular Ca2+ through the associated βγ subunits. In addition, activated CXCR4 stimulates multiple downstream signaling cascades that result in activation of Akt and extracellular signal-regulated kinases-1 and -2 (ERK-1/2), which mediate CXCR4-induced cell survival and migration (7). Previous studies indicate that CXCR4 is overexpressed in at least 23 different human cancers (8). Aberrant expression of CXCR4 appears to cause dysregulated CXCR4 trafficking and signaling that contributes to tumor growth, invasion, and metastasis (6, 9–11).

GPCR activation of Akt and ERK-1/2 occurs through both G protein-dependent and -independent mechanisms (12, 13). G protein-dependent signaling is initiated rapidly at the plasma membrane, whereas G protein-independent signaling is sustained and can occur on endosomes (14). Sustained endocytic GPCR signaling is mediated by adaptor proteins that organize signaling complexes. In many cases, organization of GPCR signaling complexes is mediated by arrestins on endosomes (15). Arrestins function as scaffolds and bind signaling molecules, such as Akt, and components of ERK-1/2 and JNK signaling cascades (13, 16). Activation of the protease-activated receptor-2 and the angiotensin AT1A receptor promotes high affinity binding of arrestin, leading to the formation of a stable complex that internalizes and sorts to endosomes together with acti

* The abbreviations used are: GPCR, G protein-coupled receptor; ESCRT, endosomal sorting complex required for transport; HEK, human embryonic kidney; ANOVA, analysis of variance; PRR, proline-rich region; SH3, Src homology 3; IB, immunoblot; IP, immunoprecipitation; pERK-1/2, phosphorylated ERK-1/2.
vated ERK-1/2 (16, 17). Activated ERK-1/2 is retained on endosomes and phosphorylates cytosolic proteins, resulting in modulation of various cellular functions. Arrestins have also been implicated in CXCR4-mediated ERK-1/2 activation, but precisely how this occurs is not known (18, 19).

Arrestins have established functions in GPCR desensitization and trafficking (20). Activated GPCRs are rapidly phosphorylated by G protein-coupled receptor kinases, which promotes arrestin binding and G protein uncoupling (21). Arrestins also facilitate GPCR internalization by interacting with components of the internalization machinery (22, 23). Similar to other GPCRs, signaling by CXCR4 is initially desensitized by G protein-coupled receptor kinase-mediated phosphorylation of the C-terminal tail serine/threonine residues, leading to arrestin binding and subsequent G protein uncoupling (7). In addition, phosphorylation of CXCR4 C-terminal tail serine residues promotes direct binding of the E3 ubiquitin ligase AIP4 (atrophin-interacting protein 4), resulting in ubiquitin modification of C-terminal tail lysine residues (24, 25). Activated CXCR4 is internalized to early endosomes and is then sorted predominately to lysosomes and degraded (26–28). Ubiquitin serves as an endosomal sorting signal for CXCR4 through interactions with components of the endosomal sorting complex required for transport (ESCRT) machinery (27, 28). The ESCRT machinery is composed of four protein complexes (ESCRT-0 to -III) that act in a sequential and coordinated manner with other factors to sort ubiquitinated cargo to intraluminal vesicles of multivesicular bodies (29, 30). Multivesicular bodies fuse with lysosomes, where intraluminal vesicles and content are degraded. We have recently shown that arrestin-2 mediates endosomal sorting of CXCR4 via interactions with AIP4 and STAM-1 (signal-transducing adaptor molecule-1), a component of ESCRT-0 (26, 27). Interestingly, the complex formed by arrestin-2 and STAM-1 negatively regulates CXCR4 degradation (27).

Despite the importance of CXCR4 signaling in development and disease, the molecular mechanisms mediating CXCR4 signaling remain poorly understood. Here, we now report that CXCR4-induced ERK-1/2 signaling is mediated by a discrete subpopulation of STAM-1 and AIP4 localized to caveolae. Our data suggest that AIP4 binding and ubiquitination of STAM-1 are required for this process. Thus, in addition to the established roles of AIP4 and STAM-1 in endocytic trafficking, we provide evidence that AIP4 and STAM-1 also function as GPCR signaling mediators. Our study identifies a new mechanism that links an E3 ubiquitin ligase and the ESCRT machinery to the regulation of GPCR signaling.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—Human embryonic kidney 293 (HEK293) (Microbiex, Toronto, Canada), HeLa (American Type Culture Collection), and SKBR3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone Laboratories (Logan, UT)) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories). Mouse embryonic fibroblast cells isolated from arrestin-2 and arrestin-3 double knock-out embryos and matched wild-type embryos were provided by Robert J. Lefkowitz (Duke University, Durham, NC). The AIP4 (D20 and G11), Gα12 (T-19), and dynamin I (D5) monomeric antibodies and c-Myc and caveolin-1 polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The β2-adaptin, CXCR4 (2B11), μ2 (AP50), PE-conjugated CXCR4 (CD184), and isotype control antibodies were from BD Biosciences. The α-adaptin antibody (clone AP6) was from Fisher. The arrestin antibody was kindly provided by Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA). The STAM-1 polyclonal antibody was from ProteinTech Group (Chicago, IL). The ERK-1/2 and phospho-ERK-1/2 (Thr-183 and Tyr-185 in ERK-2) antibodies were from Sigma. The c-Myc (9E10) monoclonal antibody was from Covance (Berkeley, CA). The T7 goat polyclonal antibody was from Abcam (Cambridge, MA). The actin antibody was from MP Biomedicals (Aurora, OH). The β-tubulin monoclonal antibody was from Accurate Chemical and Scientific (Westbury, NY). Alexa-Fluor 635-conjugated goat anti-mouse, Alexa-Fluor 594-conjugated anti-rat, Alexa-Fluor 488-conjugated goat anti-rabbit, and Alexa-Fluor 568-conjugated goat anti-rabbit antibodies were from Molecular Probes (Eugene, OR). Mounting medium for immunofluorescence microscopy was from Vector Laboratories (Burlingame, CA). Stromal cell-derived factor-1α (SDF-1α; CXCL12) and epidermal growth factor (EGF) were from PeproTech (Rocky Hill, NJ). The control (GAPDH), STAM-1 (GAACGAAGAUCGAGU AU), AIP4 (GGUGACAAAGAGGCAACAGAG), caveolin-1 (siGENOME SMARTpool M-003467-01-005), μ2 (UCAACG GCAGCCAGGU), and arrestin-2/3 (ACCUGCGCUCC CGGAUA) siRNAs were from Dharmacon RNA Technologies (Lafayette, CO).

DNA Constructs—GST-STAM-1, GST-STAM-2, GST-AIP4, YFP-STAM-1, YFP-AIP4, HA-ubiquitin, GST-AIP4 truncation mutants, FLAG-AIP4, Myc-AIP4 wild type and C830A mutant, FLAG- and Myc-STAM-1 and -2, HIS STAM-1, T7-STAM-1, FLAG-STAM-1-CC(296–380), and FLAG-Arc2(25–161) were described previously (26–28). For FLAG-AIP4-ΔPRR, the region encompassing amino acid residues 199–216 was deleted by two-step PCR with mutually annealing overlapping primers and flanking primers based on 3×FLAG-pCMV-10. Amplified product was digested and ligated into NotI and BamHI sites of 3×FLAG-pCMV-10 (GE Healthcare). For GST-STAM-1-SH3, amino acid residues 209–269 were amplified by PCR and ligated into BamHI and XhoI sites of pGEX-4T2. The sequences of all constructs were verified by dideoxysequencing. For FLAG-STAM-1-ΔSH3, the region encompassing amino acid residues 209–269 was deleted by two-step PCR. Amplified product was digested and ligated into the HindIII and XbaI sites of 3×FLAG-pCMV-10.

GST Pull-down Assay—GST fusion proteins were expressed in Escherichia coli BL21 cells and purified by immobilization on glutathione 4B-Sepharose resin, as described previously (27). HeLa cells transiently expressing FLAG-tagged AIP4, STAM-1, and/or STAM-2 were lysed in binding buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). Clarified lysates were incubated with immobilized GST, GST STAM-1, GST-STAM-2, and/or GST-AIP4, and bound proteins were eluted in 2× sample buffer by boiling for 10 min and
detected by SDS-PAGE followed by immunoblotting, essentially as we have described previously (27). For AIP4 mapping studies, equimolar amounts (186 nm) of GST-AIP4, GST-AIP4-ΔWW-I-IV, GST-AIP4-ΔPRR, and GST alone were incubated with clarified HeLa cell lysates expressing FLAG-STAM-1. Equimolar amounts (133 nm) of GST-STAM-1 and GST were incubated with clarified HeLa cell lysates expressing Myc-AIP4, Myc-AIP4-ΔPRR, and empty vector (pcDNA3). For binding experiments between AIP4 and STAM-1-SH3 domain, equimolar amounts (186 nm) of GST-STAM-1-SH3 or GST alone were incubated with 100 μl of cell lysate from HeLa cells expressing FLAG-AIP4. The binding experiment and analysis were performed as described above. For direct binding experiments, His-tagged STAM-1 was purified from E. coli BL21 cells by immobilization to HIS-Select nickel affinity beads and elution binding buffer containing 150 mM imidazole. GST-AIP4 immobilized on glutathione 4B-Sepharose resin was incubated with 500 ng of HIS-STAM-1.

CXCR4 Internalization Assay—HeLa cells grown on 10-cm dishes were treated with 50 μg/ml nystatin or vehicle (DMSO) in DMEM containing 20 mM HEPES for 30 min at 37 °C. The cells were washed twice with PBS and detached from the surface of the plate with cell disassociation solution and transferred to a tube containing 8 ml of PBS supplemented with 0.1% BSA (Media Tech). Cells were collected by centrifugation and resuspended in PBS plus 0.1% BSA, and 5 × 10^6 cells were transferred in 250 μl to a fresh tube. Cells were treated with 50 nM CXCL12 for 2, 5, 10, and 20 min and with vehicle for 20 min at 37 °C. Following treatment, 4 ml of cold PBS was added to each tube, and cells were collected by centrifugation and resuspended in 500 μl of 4% paraformaldehyde-PBS for 15 min at 37 °C for fixation. Cells were collected by centrifugation and washed three times with 4 ml of PBS. Cells were resuspended in 100 μl of PBS plus 0.1% BSA supplemented with 5% normal goat serum and stained with PE-conjugated anti-CXCR4 (1:100 dilution) or isotype control antibodies for 1 h at room temperature. Following staining, cells were washed by adding 4 ml of PBS to each tube, and the cells were collected by centrifugation and resuspended in 300 μl of PBS plus 0.1% BSA. CXCR4 surface expression was analyzed by flow cytometry (FACS-CANTO II; BD Biosciences), and analysis was performed using FlowJo version 9.3.

Cell Fractionation Experiments—HeLa cells transfected with FLAG-AIP4 grown to 100% confluence in a 10-cm dish were treated with vehicle (PBS with 0.1% BSA) and 10 nm CXCL12 for 5 min. Caveolin-1 enriched fractions were isolated using a detergent-free procedure (31). Cells were scraped into 1 ml of carbonate buffer (150 mM sodium carbonate, pH 11, 1 mM EDTA, 10 μg/ml each of leupeptin, aprotinin, and pepstatin A) and transferred to a prechilled ultracentrifuge tube. Cells were lysed by passing through a Dounce homogenizer 10 times, followed by passing through an 18-gauge needle 10 times and sonication. Eight hundred μl of lysed cells was added to an equal volume of MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) containing 80% sucrose and 300 mM sodium carbonate. Samples were pipetted to the bottom of a 12-ml prechilled ultracentrifuge tube followed by layering the top of the lysate with 6 ml of 35% sucrose in MBS buffer containing 150 mM sodium carbonate, which was then layered with 4 ml of 5% sucrose in MBS buffer. Tubes were placed in an SW41 swinging bucket rotor and centrifuged at 4 °C for 18 h at 221,000 × g. Nine sequential fractions (1.330 ml) were removed from the top, and equal volumes of each fraction were analyzed by immunoblotting to detect CXCR4, β2-adaptin, caveolin-1, STAM-1, FLAG-AIP4, and Gα13.

Confocal Fluorescence Microscopy—HeLa cells were serum-starved by incubating with DMEM containing 20 mM HEPES, pH 7.5, for 3–4 h at 37 °C. Cells were treated with 10 nM CXCL12 or vehicle for 30 min, fixed with 3.7% paraformaldehyde, and then permeabilized with 0.05% (w/v) saponin for 10 min, similar to a protocol we have described previously (26, 27). Cells were co-incubated with primary antibodies against α-adaptin, CXCR4, caveolin-1, and STAM-1 for 1 h at 37 °C, followed by incubating with appropriate Alexa-Fluor-conjugated secondary antibodies for 30 min at 37 °C. Cells were washed and mounted onto glass slides using Vectashield mounting medium containing DAPI. Samples were analyzed using a Zeiss LSM 510 laser-scanning confocal microscope equipped with a Plan-Apo ×63/1.4 numerical aperture oil lens objective. Images were acquired using a 1.4-megapixel cooled extended spectra range RGB digital camera set at 512 × 512 resolution. Acquired images were then analyzed using ImageJ software (version 1.41o) and Adobe Photoshop (CS4). To examine STAM-1, caveolin-1, and α-adaptin or AIP4, caveolin-1, and α-adaptin distribution, HeLa cells were transiently transfected with YFP-STAM-1 or YFP-AIP4, respectively.

ERK-1/2 Phosphorylation Assay—HeLa or HEK293 cells were transiently transfected with siRNA (600 pmol) targeting arrestin-2/3, AIP4, STAM-1, and GAPDH or DNA encoding dynamin-K44A, FLAG-STAM-1-CC, FLAG-arrestin-2(25–161), FLAG-AIP4-ΔPRR, FLAG-STAM-1, FLAG-AIP4, FLAG-STAM-1, Myc-AIP4-C830A, Myc-STAM-1, Myc-AIP4-WT, and Myc-STAM-1, as described previously (32). Cells were treated with 10 nM CXCL12, 100 ng/ml EGf, or 1 mM carbachol and vehicle (0.1% BSA) for several time periods. To examine the effect of nystatin on ERK-1/2 phosphorylation, HeLa cells were treated with 50 μg/ml nystatin or vehicle (DMSO) for 30 min before stimulation for 5 min. Phosphorylation of ERK-1/2 was determined by immunoblotting followed by densitometric analysis. To examine the role of caveolin-1 on CXCR4-induced ERK-1/2 phosphorylation, we employed quantitative confocal immunofluorescence microscopy. HeLa cells transfected with 600 pmol of caveolin-1 or GAPDH siRNA were stimulated with 10 nM CXCL12 or vehicle for 5 min at 37 °C. Following treatment, cells were washed, fixed, and co-stained with anti-phospho-ERK-1/2 (pERK-1/2) mouse monoclonal and anti-caveolin-1 rabbit polyclonal antibodies overnight at 4 °C. Cells were washed and stained with Alexa-Fluor-conjugated secondary antibodies for 1 h at room temperature. Images were acquired as described above. The mean pixel intensity of pERK-1/2 and caveolin-1 staining per cell was calculated using LSM 510 image software, and the average of the mean pixel intensity from 45 cells from three independent experiments was determined.

Ubiquitination and Co-immunoprecipitation Assays—For the ubiquitination assay, HeLa cells were transfected with FLAG-STAM-1 or empty vector (pCMV-10), Myc-AIP4 or
Myc-AIP4-C830A, and HA-ubiquitin or empty vector (pcDNA3). To examine the effect of agonist on STAM-1 ubiquitination, cells were co-transfected with T7-STAM-1 and HA-ubiquitin. Cells were serum-starved and treated with 100 nM CXCL12 for 5 min. STAM-1 was immunoprecipitated, and ubiquitinated species were detected by immunoblotting. For the co-immunoprecipitation assay, HeLa cells were transfected with Myc-AIP4 and FLAG-STAM-1 together or alone with empty vector (pcDNA). To detect binding between endogenous proteins, HeLa cells, HEK293 cells stably expressing HA-CXCR4 and BT474 cells were used. These experiments were essentially performed as described previously (27).

**Statistical Analysis**—Data were analyzed by Student’s t test or one-way or two-way analysis of variance (ANOVA) using GraphPad Prism 4.0 for Macintosh (GraphPad Software, San Diego, CA).

**RESULTS**

**STAM-1 and AIP4 Are Required for CXCR4-induced ERK-1/2 Activation**—We recently showed that arrestin-2 localizes to endosomes and regulates sorting of CXCR4 to the degradative pathway via interactions with AIP4 and STAM-1. Arrestin-2 has also been linked to CXCR4-induced ERK-1/2 activation in HEK293 cells (18, 19), but whether STAM-1 and/or AIP4 function in this pathway is not known. In the present study, we sought to determine whether the CXCR4 sorting machinery regulates its signaling responses in HeLa cells, which express endogenous CXCR4 (27). We first examined the role of arrestins in CXCR4-induced ERK-1/2 activation. HeLa cells transfected with siRNAs targeting arrestin-2 and -3 were treated with CXCL12 for various times, and phosphorylation of ERK-1/2 was determined by immunoblotting. As shown in Fig. 1A,
CXCL12 induced a rapid and transient increase in ERK-1/2 activation in control cells transfected with siRNA against GAPDH, which was similar to that observed in either mock or untransfected HeLa cells (supplemental Fig. S1A). The kinetics of ERK-1/2 activation was similar in cells transfected with siRNA against arrestin-2/3; however, the magnitude of the response was significantly increased in the absence of arrestin-2 and arrestin-3 expression. We also observed that CXCR4-induced ERK-1/2 activation was enhanced in mouse embryonic fibroblast cells isolated from arrestin-2/3 knock-out mice (supplemental Fig. S2A). These findings suggest that termination of CXCR4 signaling is defective in cells lacking arrestins and is consistent with a role for arrestins in CXCR4 desensitization (7).

We next examined the function of STAM-1 and AIP4 in CXCR4-induced ERK-1/2 activation. In contrast to arrestin, depletion of STAM-1 significantly attenuated CXCR4-induced ERK-1/2 activation, as compared with control HeLa cells transfected with GAPDH siRNA (Fig. 1B). Similar results were observed in HEK293 cells expressing HA-CXCR4 (supplemental Fig. S2B). Remarkably, AIP4 depletion also significantly attenuated CXCR4-induced ERK-1/2 activation (Fig. 1C). Importantly, CXCR4 surface levels were not altered in cells treated with siRNA against STAM-1 or arrestin-2/3, although they were modestly increased in AIP4-depleted cells (supplemental Fig. S3). Activation of endogenous acetylcholine muscarinic receptors with carbachol also resulted in decreased ERK-1/2 activation in AIP4-deficient cells compared with control cells (supplemental Fig. S4A), indicating that AIP4 regulates signaling of other GPCRs. In contrast, epidermal growth factor-induced ERK-1/2 activation was not affected (supplemental Fig. S4B). Taken together, our data suggest that STAM-1 and AIP4, but not arrestin-2/3, are critical mediators of CXCR4-induced ERK-1/2 activation.

**STAM-1 Interacts Directly with AIP4**—To determine the mechanism by which STAM-1 and AIP4 mediate CXCR4 activation of ERK-1/2, we first examined whether these proteins associate with each other in cells. HeLa cells expressing FLAG-AIP4 were lysed and incubated with bacterially purified GST-STAM-1, GST-STAM-2, or GST immobilized on glutathione-Sepharose resin. The interaction of FLAG-AIP4 with GST fusion proteins was determined by immunoblotting. Both GST-STAM-1 and GST-STAM-2, but not GST, bound to FLAG-AIP4, suggesting that AIP4 interacts with both STAM-1 and STAM-2 (Fig. 2, A and B). Reciprocal pull-down experiments in which purified GST-AIP4 was incubated with lysates prepared from HeLa cells expressing FLAG-STAM-1 or FLAG-STAM-2 were performed and indicate that both STAM-1 and STAM-2 interact with AIP4 (Fig. 2C). To determine if these proteins interact directly, GST-AIP4 was incubated with purified His-tagged STAM-1 and revealed that STAM-1 binds directly with AIP4 (Fig. 1D). In addition, FLAG-tagged STAM-1 expressed in cells co-immunoprecipitated with Myc-tagged AIP4 (Fig. 2E), indicating that AIP4 and STAM-1 exist as part of a complex in cells. Interestingly, a high molecular weight form of FLAG-STAM-1 was evident in longer exposures of the immunoblot, suggesting that STAM-1 is post-translationally modified (Fig. 2E). To test the possibility that STAM-1 is ubiquitinated, we examined the ubiquitination status of FLAG-STAM-1 in HeLa cells co-transfected with Myc-tagged wild-type AIP4 and the AIP4 C830A catalytically inactive mutant (28). As shown in Fig. 2F, co-expression of wild-type AIP4 enhanced ubiquitination of FLAG-STAM-1, which was not evident in cells expressing the AIP4 C830A mutant, suggesting that AIP4 mediates ubiquitination of STAM-1.

**SH3 Domain in STAM-1 Interacts with Proline-rich Region in AIP4**—To define the domain important for STAM-1 interaction with AIP4, we examined the ability of STAM-1 to bind to various AIP4 deletion mutants (Fig. 3A). AIP4 contains an N-terminal C2 domain, proline-rich region (PRR), four WW domains, and HECT domain that mediates ubiquitin transfer to substrates (33). The AIP4 WW domains interact with PPXY and PPPY motifs in substrate proteins but can also interact with non-canonical PY motifs, as we have recently shown (24, 34). As shown in Fig. 3B, FLAG-STAM-1 expressed in HeLa cells bound to full-length AIP4 and to the AIP4 deletion mutant in which the WW domains were removed (∆WW-1–IV) but not to the WW domains alone and the HECT domain. These data indicate that the AIP4-STAM-1 interaction is not mediated by the WW domains, and the site of interaction probably resides within the N-terminal region of AIP4. The N terminus of AIP4 contains a C2 domain, important for phospholipid binding (35–37), and a proline-rich region. The proline-rich region of AIP4 has been shown to bind to a subset of SH3 domains (38). SH3 domains are composed of ~60 amino acids and bind to PXXP motifs in proteins (39). As shown in Fig. 3C, binding of AIP4 proline-rich region deletion mutant (∆PRR) to GST-STAM-1 was substantially diminished as compared with full-length AIP4, suggesting that the proline-rich region represents the major binding site for the STAM-1 interaction with AIP4. Interestingly, deletion of the SH3 domain in STAM-1 caused an approximately 50% reduction in binding to AIP4, indicating that the SH3 domain is necessary for binding to AIP4 (Fig. 3D). This also indicates that AIP4 interacts with other regions of STAM-1, which is consistent with what has been observed for binding between the yeast orthologs of AIP4 (i.e., Rsp 5) and STAM-1 (i.e., Hse1) (64). Nevertheless, the SH3 domain is sufficient for binding to AIP4 (Fig. 3E). Taken together, these findings suggest that the AIP4-STAM-1 interaction is mediated in part by the SH3 domain of STAM-1 and the proline-rich region of AIP4.

**AIP4 Interacts with STAM-1 to Mediate CXCR4-induced ERK Signaling**—To determine whether the interaction between AIP4 and STAM-1 regulates CXCR4-induced ERK-1/2 activation, AIP4 wild type or the proline-rich ∆PRR mutant and STAM-1 were co-expressed in HeLa cells, and CXCR4-induced ERK-1/2 activation was examined. Overexpression of wild-type AIP4 led to a significant increase in ERK-1/2 activation compared with vector-transfected control cells (Fig. 4A). Remarkably, overexpression of ∆PRR mutant failed to enhance ERK-1/2 activation, suggesting that the interaction between AIP4 and STAM-1 is necessary for CXCR4-induced ERK-1/2 activation. We next assessed whether AIP4 catalytic activity was required for this effect. As shown in Fig. 4B, the AIP4 C830A catalytically inactive mutant failed to enhance ERK-1/2 activation compared with wild-type AIP4, suggesting that the
ubiquitin ligase activity of AIP4 is necessary for CXCR4-induced ERK-1/2 activation. We next examined whether STAM-1 is ubiquitinated. Activation of CXCR4-promoted an increase in STAM-1 ubiquitination at 5 min, a time in which ERK-1/2 activation is maximal, suggesting that STAM-1 ubiquitination may be required for this process (Fig. 4C).

CXCR4 Internalization Is Not Required for ERK Signaling—We previously showed that activated CXCR4 co-localizes with AIP4 and STAM-1 on endosomes, suggesting that internalization is not required for ERK signaling.
tion of CXCR4 may be required for ERK-1/2 activation. However, co-expression of dynamin K44A, a dominant-negative mutant of dynamin that blocks CXCR4 internalization (40), had no effect on CXCR4-induced ERK-1/2 activation, suggesting that CXCR4 internalization is not required for ERK-1/2 activation (Fig. 5A). To confirm these findings, we examined the role of AP-2, a heterotetrameric protein complex that facilitates GPCR internalization through clathrin-coated pits (23, 41) and co-localizes with activated CXCR4 (28). We first evaluated CXCR4 internalization in HeLa cells treated with siRNA targeting the /H9262 subunit of AP-2. Agonist-induced internalization of endogenous CXCR4 was significantly reduced in /H9262 siRNA-transfected cells compared with control GAPDH siRNA-treated cells, indicating that AP-2 is required for CXCR4 internalization (Fig. 5B). Next, we examined CXCR4-induced ERK-1/2 activation in the same cells that were used for...
the internalization experiments. As shown in Fig. 5C, depletion of AP-2 did not affect CXCR4-induced ERK-1/2 activation compared with control siRNA-transfected cells, suggesting that CXCR4 internalization is not required for ERK-1/2 activation.

Because CXCR4 internalization is not required for ERK-1/2 activation, it is likely that molecular events proximal to the receptor at the plasma membrane mediate ERK-1/2 activation. We previously showed that CXCR4 co-localizes with AIP4 on endosomes and at the plasma membrane, but whether STAM-1 is also present is not known. We therefore examined the subcellular distribution of CXCR4 and STAM-1 by confocal immunofluorescence microscopy. To ensure that localization occurred at the plasma membrane, we co-stained cells for AP-2, which is found predominantly at the plasma membrane (42). HeLa cells transfected with T7-STAM-1 and HA-ubiquitin were treated with vehicle (PBS with 0.01% BSA) or 10 nM CXCL12 for 5 min. Samples were subjected to IP (T7 and IgG control) and analyzed by IB to detect incorporated HA-ubiquitin. Shown are representative blots from one of three independent experiments.

FIGURE 4. The role of AIP4 in CXCR4-induced ERK-1/2 phosphorylation. A and B, HeLa cells were transfected with expression constructs of STAM-1, AIP4, the proline-rich region deletion mutant (ΔPRR) (A), or the catalytically inactive mutant CB30A mutant (B). Cells were treated with vehicle (PBS with 0.01% BSA) or 10 nM CXCL12 for 5 min. The degree of ERK-1/2 phosphorylation (pERK-1/2) was determined by IB analysis and quantified by densitometry and normalization to total ERK-1/2 levels. Bars, average pERK-1/2 levels ± S.E. (error bars) from three independent experiments, expressed as fold increase in CXCL12 compared with vehicle-treated cells normalized to pERK-1/2 levels in empty vector-transfected cells. Data were analyzed by a one-way ANOVA followed by Bonferroni’s post hoc test. pERK-1/2 levels were significantly different between wild-type AIP4 and mutant AIP4-expressing cells in A and B. * , p < 0.05. C, HeLa cells transfected with T7-STAM-1 and HA-ubiquitin were treated with vehicle (PBS with 0.01% BSA) or 10 nM CXCL12 for 5 min. Samples were subject to IP (T7 and IgG control) and analyzed by IB to detect incorporated HA-ubiquitin. Shown are representative blots from one of three independent experiments.
FIGURE 5. The role of endocytosis in CXCR4-induced ERK-1/2 phosphorylation. A, HeLa cells transfected with dynamin-K44A and empty vector (pcDNA) were treated with 10 nM CXCL12 for the indicated times. pERK-1/2 levels were determined by IB analysis and quantified as described in the legend to Fig. 1. Data were analyzed by a two-way ANOVA followed by Bonferroni’s post hoc test and were found not to be statistically significant. B, HeLa cells were transfected with μ2 siRNA and treated with vehicle and 10 nM CXCL12 for 20 min. Endogenous CXCR4 cell surface levels were quantified by FACS analysis, as described under “Experimental Procedures.” Data represent the mean from four independent experiments. Error bars, S.E. Data were analyzed by Student’s t test (*, p < 0.05). C, CXCL12-promoted ERK-1/2 phosphorylation was determined in the same cells used in the experiments described in B. Data were analyzed by Student’s t test and were not found to be statistically significant.

FIGURE 6. STAM-1 co-localizes with CXCR4 at the plasma membrane. HeLa cells grown on coverslips were treated with 10 nM CXCL12 for 5 min. Cells were then fixed, permeabilized, and stained with anti-CXCR4 (red), anti-STAM-1 (green), and anti-AP2 (blue). The arrows point to puncta that show co-localization between CXCR4 and AP2. The arrowheads point to puncta that show co-localization between CXCR4 and STAM-1. The white line indicates puncta that show co-localization between CXCR4, AP2, and STAM-1. The circles with the circle indicate STAM-1 puncta that do not contain CXCR4 and AP2. A differential interference contrast (DIC) image is shown. A representative area (boxed) from each image is enlarged 4× and shown below in the bottom panels. Shown are representative micrographs from three independent experiments. Bars, 20 μm.
calized with CXCR4 in puncta that appeared to be devoid of AP-2 (Fig. 6, arrows), suggesting that CXCR4 and STAM-1 exist in microdomains at the plasma membrane that are distinct from clathrin-coated pits.

CXCR4 is known to partition into lipid rafts, microdomains enriched in cholesterol and sphingolipids, raising the possibility that CXCR4 and STAM-1 co-localize to this compartment. Caveolae are a type of lipid raft that contain caveolin-1, a protein essential for caveolae formation (44). Many GPCRs and signaling effectors have been shown to localize caveolae (44). Therefore, we examined the distribution of CXCR4, STAM-1, and AIP4 with caveolin-1 by sucrose density fractionation (31). HeLa cells were treated with or without CXCL12 for 5 min, lysed, and subjected to sucrose gradient fractionation. Caveolin-1 was used as a marker of caveolae, whereas \( \beta_2 \)-adaptin, a subunit of AP-2, is a marker of heavy fractions. In both untreated and treated CXCL12 cells, CXCR4 was present in fractions enriched with \( \beta_2 \)-adaptin (Fig. 7A), consistent with the immunofluorescence microscopy data that showed that CXCR4 co-localizes with AP-2 (Fig. 6). CXCR4 was also present in caveolin-1 enriched fractions in control and agonist-treated cells, suggesting that CXCR4 localizes to caveolae. CXCR4 is known to couple to \( G_\gamma \) (7), which co-fractionates with CXCR4 in both enriched and non-enriched caveolin-1 fractions (Fig. 7A). We next examined the distribution of AIP4 and STAM-1. To circumvent issues detecting endogenous AIP4, HeLa cells were transfected with a low amount of FLAG-AIP4 and were treated with vehicle (PBS with 0.01% BSA) and 10 nM CXCL12 for 5 min. Sucrose gradient centrifugation was performed, as described under “Experimental Procedures.” Nine fractions were collected and subjected to IB analysis for the presence of caveolin-1, CXCR4, FLAG-AIP4, STAM-1, \( G_\gamma \), and \( \beta_2 \)-adaptin. Shown are representative blots from one of three experiments.

![Figure 7. CXCR4 co-fractionates with AIP4, STAM-1, and caveolin-1. A, HeLa cells were transfected with low amounts of FLAG-AIP4 and were treated with vehicle (PBS with 0.01% BSA) and 10 nM CXCL12 for 5 min. Sucrose gradient centrifugation was performed, as described under “Experimental Procedures.” Nine fractions were collected and subjected to IB analysis for the presence of caveolin-1, CXCR4, FLAG-AIP4, STAM-1, \( G_\gamma \), and \( \beta_2 \)-adaptin. Shown are representative blots from one of three experiments. Arrows, STAM-1. *, heavy chain of IgG.](image-url)

![Figure 7A.](image-url)

![Figure 7B.](image-url)

![Figure 7C.](image-url)

FIGURE 7. CXCR4 co-fractionates with AIP4, STAM-1, and caveolin-1. A, HeLa cells were transfected with low amounts of FLAG-AIP4 and were treated with vehicle (PBS with 0.01% BSA) and 10 nM CXCL12 for 5 min. Sucrose gradient centrifugation was performed, as described under “Experimental Procedures.” Nine fractions were collected and subjected to IB analysis for the presence of caveolin-1, CXCR4, FLAG-AIP4, STAM-1, \( G_\gamma \), and \( \beta_2 \)-adaptin. Shown are representative blots from one of three experiments. Arrows, STAM-1. *, heavy chain of IgG.

To confirm that CXCR4, STAM, and AIP4 are present in caveolin-1 enriched microdomains, we performed immunofluorescence confocal microscopy. Because caveolin-1 localizes to endosomes (45), cells were co-stained for AP-2 to ensure that only caveolin-1 at the plasma membrane was visualized in these experiments. Similar to the sucrose fractionation data, we found that CXCR4, YFP-AIP4, and YFP-STAM-1 co-localized with caveolin-1 at the plasma membrane (supplemental Figs. S5–S7). We next examined whether caveolin-1 exists in a complex with AIP4 and STAM-1 in cells. HEK293 (Fig. 7B) or HeLa (Fig. 7C) cells were lysed, endogenous AIP4 was immunoprecipitated, and co-association of endogenous caveolin-1 and STAM-1 was determined by immunoblot. Caveolin-1 and STAM-1 were found to co-immunoprecipitate with AIP4 and not IgG control, suggesting that AIP4 and STAM-1 exist in a complex with caveolin-1 in cells (Fig. 7, B and C). Taken together, these findings suggest that CXCR4, STAM-1, and AIP4 associate at the plasma membrane in caveolar microdomains.

STAM-1 and AIP4 Mediate CXCR4 Signaling

9022 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 287 • NUMBER 12 • MARCH 16, 2012
Caveolin-1 Is Required for CXCR4-induced ERK-1/2 Activation

To confirm a role for caveolae in CXCR4-induced ERK-1/2 activation, we first used the cholesterol-sequestering drug nystatin, which disrupts caveolae and lipid rafts (46). HeLa cells were preincubated with nystatin for 30 min at 37 °C and then treated with CXCL12 for 5 min. CXCL12-induced ERK-1/2 activation was significantly inhibited in nystatin-treated cells (Fig. 8A). In contrast, nystatin pretreatment failed to affect EGFR-induced ERK-1/2 activation, suggesting that nystatin does not globally affect signaling from the plasma membrane (Fig. 8A). Moreover, the initial rate of CXCR4 internalization was similar in control and nystatin-treated cells and only modestly attenuated at later times in cells exposed to nystatin (supplemental Fig. S8). To determine whether nystatin affected CXCR4-induced ERK-1/2 activation in other cell types, we used SKBR3 cells, a breast cancer cell line that expresses high levels of endogenous CXCR4. Similar to HeLa cells, nystatin significantly inhibited CXCR4-induced ERK-1/2 activation in SKBR3 cells (supplemental Fig. S9), whereas ERK-1/2 activation induced by EGFR remained intact (supplemental Fig. S7A). These findings suggest that CXCR4-induced ERK-1/2 activation occurs in caveolae or lipid rafts.

To determine whether disruption of caveolae per se affected CXCR4-induced ERK-1/2 activation, we used siRNA to deplete Caveolin-1 and AIP4 Mediate CXCR4 Signaling

STAM-1 and AIP4 Mediate CXCR4 Signaling
cells of caveolin-1, the major structural component of caveolae (44). HeLa cells transfected with caveolin-1-specific siRNA showed almost complete loss of caveolin-1 expression as assessed by immunoblot and immunofluorescence microscopy as compared with siRNA-transfected control cells (Fig. 8, B and E). We examined ERK-1/2 activation by quantitative confocal immunofluorescence microscopy, which enabled us to focus on individual cells and to determine the subcellular distribution of activated ERK-1/2 in cells. HeLa cells were treated with CXCL12 or vehicle for 5 min, fixed, and co-stained for caveolin-1 and phosphorylated ERK-1/2. In control siRNA-transfected cells, CXCL12 caused a significant increase in phosphorylated ERK-1/2 staining, which was mostly nuclear, as compared with vehicle-treated cells (Fig. 8, B and C). In contrast, in caveolin-1 siRNA-transfected cells, phosphorylated ERK-1/2 staining was significantly attenuated, as compared with vehicle-treated cells. Taken together, these data suggest that caveolae are important for CXCR4-induced activation of ERK-1/2.

DISCUSSION

In the present study, we delineated a novel molecular mechanism by which the chemokine receptor CXCR4 promotes activation of the ERK-1/2 signaling cascade. We discovered a new function for the E3 ubiquitin ligase AIP4 and the ESCRT-0 protein STAM-1 in CXCR4 signaling, which is distinct from their established role in receptor degradation and signal termination. We previously showed that AIP4 and STAM-1 mediate sorting of CXCR4 from endosomes to lysosomes for degradation (27). In the present study, we describe a new function for AIP4 and STAM-1 in CXCR4 signaling that appears to be limited to a subpopulation of CXCR4 present in caveolar microdomains. We further show that AIP4 binds to and ubiquitinates STAM-1 following agonist stimulation and that the AIP4-STAM-1 interaction is necessary for CXCR4-induced ERK-1/2 activation. Our data are consistent with the notion that spatial segregation of AIP4 and STAM-1 in caveolae is required for CXCR4 signaling. This mechanism may be applicable to other GPCRs because depletion of AIP4 also attenuated muscarinic receptor-induced ERK-1/2 activation (supplemental Fig. S4A).

The interaction between AIP4 and STAM-1 as well as AIP4 ubiquitin ligase activity are important for CXCR4-induced ERK-1/2 activation (Fig. 4, A and B). AIP4 binding to STAM-1 may activate and/or enhance AIP4 ligase activity. AIP4 exists in an autoinhibitory conformation that occurs via an intramolecular interaction between the HECT domain and the WW domains (47). Phosphorylation of serine/threonine residues within the proline-rich region releases the intramolecular inhibition, thereby enabling AIP4 to interact with and ubiquitinate target substrates. STAM-1 binding to AIP4 may alleviate the autoinhibitory state, enabling ubiquitination of target substrates. STAM-1 is also ubiquitinated by AIP4 (Fig. 2F), raising the possibility that STAM-1 ubiquitination is important for CXCR4-induced ERK-1/2 activation, although this remains to be determined. STAM-1 is required for CXCR4 endosomal sorting, and STAM-1 is not degraded upon CXCR4 activation (27), suggesting that ubiquitination of STAM-1 has a unique function. Interestingly, STAM-1 also binds to the deubiquitinating enzymes associated molecule with the SH3 domain of STAM (AMSH) and USP8 via its SH3 domain (48), but whether they also regulate CXCR4 signaling remains to be determined. We have recently shown that associated molecule with the SH3 domain of STAM (AMSH) does not mediate agonist-induced degradation of CXCR4 (27). Thus, our study suggests that an ESCRT protein regulates GPCR-stimulated ERK-1/2 signaling at the plasma membrane.

Ubiquitin and arrestins have been previously shown to regulate in GPCR-induced ERK-1/2 signaling. Ubiquitination of arrestin-3 by the E3 ligase Mdm2 induces a stable association between arrestin-3 and phosphorylated ERK-1/2 following activation of a subset of GPCRs (49). GPCRs co-internalize with ubiquitinated arrestin-3 and bind to and facilitate ERK-1/2 signaling from endosomes (15). We have previously shown that arrestins interact with STAM-1. However, our present study indicates that arrestins do not mediate CXCR4-induced ERK-1/2 activation (Fig. 1A and supplemental Fig. S2A). It was recently reported that siRNA-mediated depletion of arrestin-2 (and perhaps to a lesser degree arrestin-3) attenuated CXCR4-induced ERK-1/2 activation in HEK293 cells, suggesting that arrestin-2 regulates CXCR4-mediated ERK-1/2 signaling (18, 19). This discrepancy with our study is probably a consequence of differences in experimental design because we performed our experiments using HeLa cells in which both non-visual arrestins were depleted simultaneously. Nevertheless, we do not believe that arrestin-2 interacts with STAM-1 to modulate CXCR4-induced ERK-1/2 activation because expression of minigenes that disrupt this interaction (27) does not affect CXCR4-induced ERK-1/2 activation (supplemental Fig. S11).

Our findings indicate that CXCR4-mediated ERK-1/2 signaling occurs at the plasma membrane. CXCR4-induced ERK-1/2 activation is rapid and transient, reaching a maximum response at 5 min following agonist exposure, and returns to basal levels by 10 min (supplemental Fig. S1A). In contrast to CXCR4, the angiotensin AT_1A receptor induces a rapid increase in ERK-1/2 activation that is sustained for up to 90 min following agonist stimulation (12). The early and late phases of ERK-1/2 activation are mechanistically distinct, occurring through G protein-dependent and -independent mechanisms. The early phase is G protein-dependent and leads to nuclear localization of pERK-1/2, whereas the later sustained signal is arrestin-dependent and results in ERK-1/2 signaling from endosomes (12). The transient nature of ERK-1/2 activation induced by CXCR4 and the rapid appearance of ERK-1/2 in the nucleus following receptor activation (Fig. 8) is consistent with G protein-dependent signaling occurring at the plasma membrane. Indeed, we found that CXCR4-induced ERK-1/2 signaling is completely blocked by pretreatment with pertussis toxin, which inactivates Gβγi/o (data not shown). In addition, CXCR4 does not induce sustained ERK-1/2 activation (supplemental Fig. S1), and inhibition of CXCR4 endocytosis has no effect on ERK-1/2 activation (Fig. 5), arguing against an endosome-associated signaling event. However, this does not exclude the possibility that an endosome-associated signaling event occurs rapidly through second messenger signaling and/or possibly some other mechanism. One function of nuclear ERK is to stimulate cellular proliferation. However, depletion of AIP4 and STAM-1 by
siRNA did not affect CXCR4-induced proliferation of cells (supplemental Fig. S10). Therefore, the functional consequence of CXCR4-induced AIP4/STAM-1-dependent ERK-1/2 activation remains to be elucidated.

Our data are consistent with the idea that segregation of CXCR4 and/or its signaling mediators at plasma membrane microdomains is required for ERK-1/2 activation. Depletion of caveolin-1 by siRNA or treatment of cells with the cholesterol-sequestering drug nystatin attenuated CXCR4-induced ERK-1/2 activation (Fig. 8). Caveolae are a subset of lipid rafts that contain caveolin proteins and function as microdomains that organize signaling effectors (44). Caveolin-1 is thought to scaffold components of G protein signaling pathways, including heterotrimeric G proteins and their effector molecules (50). In this study, we used several approaches to show that CXCR4 as well as AIP4 and STAM-1 localize to caveolae. We found that CXCR4, its cognate G protein Go(i), and AIP4 and STAM-1 co-fractionate with caveolin-1 in buoyant membrane fractions isolated by sucrose gradient centrifugation (Fig. 7A). However, we failed to observe any marked change in distribution of these proteins in control versus CXCL12-treated cells, suggesting that they are constitutively localized in caveolae. Consistent with this notion, AIP4 and STAM-1 basally interact with caveolin-1 in cells, as determined by co-immunoprecipitation (Fig. 7, B and C). We also showed that CXCR4, AIP4, and STAM-1 co-localize with caveolin-1 at the plasma membrane as determined by confocal fluorescence microscopy (supplemental Figs. S5–S7), although this is observed in only a minor fraction of puncta. Taken together, these data suggest that CXCR4, AIP4, and STAM-1 are localized to caveolae. However, CXCR4, STAM-1, and AIP4 also co-fractionate with heavy membrane fractions (Fig. 7A) and co-localize with AP-2 (supplemental Figs. S5–S7). This is consistent with their presence in non-caveolar membrane domains, such as clathrin-coated pits and/or endosomal membranes. However, we showed that neither CXCR4 internalization nor AP-2 is required for ERK-1/2 activation (Fig. 5). Our data are consistent with the idea that a discrete subpopulation of AIP4 and STAM-1 localized to caveolae is essential for CXCR4-induced ERK-1/2 activation. It is possible that caveolae-localized AIP4 and STAM-1 facilitate or stabilize CXCR4/G protein interactions to promote ERK-1/2 activation.

The determinants that specify AIP4 and STAM-1 segregation to caveolae are not known. AIP4 belongs to the Ned4 family of E3 ubiquitin ligases that contain an N-terminal C2 domain (52). The C2 domain of AIP4 is important for membrane targeting (38, 53). The C2 domain of other members of the Ned4-like family has been shown to bind to membrane lipids (35, 37). Interestingly, caveolin-1 was co-immunoprecipitated with AIP4 and STAM-1, suggesting that AIP4 and STAM-1 may be scaffolded by caveolin-1 and thus may be recruited to caveolae through this interaction (Fig. 7, B and C). A more detailed study will be required to establish whether these interactions are modulated by CXCR4 activation. Lipid rafts have been implicated in CXCR4 signaling in T cell lines and in prostate cancer cell lines where CXCR4-induced trans-activation of the receptor tyrosine kinase HER2 promotes Akt activation, which contributes to tumor invasiveness (43, 54, 55). Therefore, segregation of CXCR4 into caveolae and/or lipid rafts may be required for signaling to distinct pathways and may contribute to CXCR4 function in cancer and possibly other diseases. It remains to be determined if AIP4 and/or STAM-1 contribute to CXCR4 signaling in these contexts.

Interestingly, AIP4 has been shown to interact with Jun N-terminal kinase 1 (JNK1) via a MAPK docking domain located within its HECT domain (47). T cell receptor-induced JNK1-mediated phosphorylation of AIP4 leads to ubiquitination and proteasomal degradation of the Jun transcription factor, which down-regulates cytokine production in T cells (56). AIP4 may also negatively regulate JNK signaling following sorbitol-induced stress by mediating ubiquitination and degradation of MKK4, the upstream activator of JNK (57). Our data presented here are consistent with a role for AIP4 in positively regulating ERK-1/2 activation induced by a GPCR. Although the precise mechanism by which AIP4 functions in this process is unknown, AIP4 interaction with STAM-1 is required. STAM-1 is a component of ESCRT-0 and functions in targeting ubiquitinated cargo to the degradative pathway. However, STAM-1 and STAM-2 were originally identified as substrates for tyrosine phosphorylation downstream of several cytokine and growth factor receptors (58–61) and were shown to function in cytokine-induced T-cell development and survival, possibly through an interaction with Janus kinases (62). However, STAM-1 and -2 function in regulation of ERK-1/2, and Akt activation has not been previously described to our knowledge. Thus, our study reveals a new role for AIP4 and STAM-1 in GPCR-induced ERK-1/2 signaling, and whether other components of the ESCRT machinery are also involved remains to be determined.

In summary, our study reveals functions for AIP4 and STAM-1 in cell signaling that are different from their established roles in endosomal trafficking. Here, we show that AIP4 binding to and possibly ubiquitination of STAM-1 mediates CXCR4-induced ERK-1/2 activation. Our data reveal that a subpopulation of AIP4 and STAM-1 co-localize with caveolin-1 in cells and that caveolae are required for CXCR4-induced ERK-1/2 activation. We propose a model in which AIP4-induced ubiquitination of STAM-1 mediates CXCR4-stimulated ERK-1/2 signaling. CXCR4 mediates cancer cell metastasis and tumor growth, and ERK-1/2 signaling probably contributes to this process (7, 51, 63). Therefore, the identification of AIP4 and STAM-1 as mediators of CXCR4-induced ERK-1/2 activation in this study may be useful for developing new therapeutic approaches for diseases involving CXCR4.

REFERENCES

1. Agarwal, U., Ghalayini, W., Dong, F., Weber, K., Zou, Y. R., Rabhany, S. Y., Rafii, S., and Penn, M. S. (2010) Role of cardiac myocyte CXCR4 expression in development and left ventricular remodeling after acute myocardial infarction. Circ. Res. 107, 667–676
2. Ceredini, D. J., Kulkarni, A. R., Callaghan, M. J., Tepper, O. M., Bastidas, N., Kleinman, M. E., Capla, J. M., Galiano, R. D., Levine, J. P., and Gurtner, G. C. (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat. Med. 10, 858–864
3. Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., and Springer, T. A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and delayed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 95, 9448–9453
atrophin-interacting protein 4 binds directly to the chemokine receptor CXCR4 via a novel WW domain-mediated interaction. *Mol. Biol. Cell* **20**, 1324–1339
25. Marchese, A., and Benovic, J. L. (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J. Biol. Chem.* **276**, 45509–45512
26. Bhandari, D., Trejo, J., Benovic, J. L., and Marchese, A. (2007) Arrestin-2 interacts with the ubiquitin-protein isopeptide ligase atrophin-interacting protein 4 and mediates endosomal sorting of the chemokine receptor CXCR4. *J. Biol. Chem.* **282**, 36971–36979
27. Malik, R., and Marchese, A. (2010) Arrestin-2 interacts with the endosomal sorting complex required for transport machinery to modulate endosomal sorting of CXCR4. *Mol. Biol. Cell* **21**, 2529–2541
28. Marchese, A., Rabborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (2003) The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev. Cell* **5**, 709–722
29. Hurley, J. H., and Hanson, P. I. (2010) Membrane budding and scission by the ESCRT machinery. It's all in the neck. *Nat. Rev. Mol. Cell Biol.* **11**, 556–566
30. Hurley, J. H., and Stenmark, H. (2011) Molecular mechanisms of ubiquitin-dependent membrane traffic. *Annu. Rev. Biophys.* **40**, 119–142
31. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* **271**, 9690–9697
32. Wyatt, D., Malik, R., Vesecky, A. C., and Marchese, A. (2011) Small ubiquitin-like modifier modification of arrestin-3 regulates receptor trafficking. *J. Biol. Chem.* **286**, 3884–3893
33. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2563–2567
34. Macias, M. J., Wiesner, S., and Sudol, M. (2002) WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. *FEBS Lett.* **513**, 30–37
35. Dunn, R., Klos, D. A., Adler, A. S., and Hicke, L. (2004) The C2 domain of ArfGAPs is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its endosome-lysosome targeting domains. Structural basis and implications for cellular signal transduction. *Biochem. J.* **390**, 641–653
36. Orsini, M. J., Parent, J. L., Mundell, S. J., Marchese, A., and Benovic, J. L. (2000) Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the C-terminal tail that mediate receptor internalization *J. Biol. Chem.* **275**, 25876
37. Kim, Y. M., and Benovic, J. L. (2002) Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. *J. Biol. Chem.* **277**, 30760–30768
38. Robinson, M. S. (1987) 100-kDa coated vesicle proteins. Molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* **104**, 887–895
39. Nguyen, D. H., and Taub, D. (2002) CXCR4 function requires membrane cholesterol. Implications for HIV infection. *J. Immunol.* **168**, 4121–4126
40. Patel, H. H., Murray, F., and Insel, P. A. (2008) Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu. Rev. Pharmacol. Toxicol.* **48**, 359–391
41. Hayer, A., Stoeber, M., Ritz, D., Engel, S., Meyer, H. H., and Helenius, A. (2010) Caveolin-1 is ubiquitinated and targeted to intraluminal vesicles in...
endolysosomes for degradation. J. Cell Biol. 191, 615–629

46. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium. Reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. J. Cell Biol. 127, 1217–1232

47. Gallagher, E., Gao, M., Liu, Y. C., and Karin, M. (2006) Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. Proc. Natl. Acad. Sci. U.S.A. 103, 1717–1722

48. Kato, M., Miyazawa, K., and Kitamura, N. (2000) A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP. J. Biol. Chem. 275, 37481–37487

49. Shenoy, S. K., Barak, L. S., Xiao, K., Ahn, S., Berthouze, M., Shukla, A. K., Luttrell, L. M., and Lefkowitz, R. J. (2007) Ubiquitination of /H9252-arrestin links seven-transmembrane receptor endocytosis and ERK activation. J. Biol. Chem. 282, 29549–29562

50. Oh, P., and Schnitzer, J. E. (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. Gq binds caveolin to concentrate in caveolae, whereas Gi and Gs target lipid rafts by default. Mol. Biol. Cell 12, 685–698

51. Yu, T., Wu, Y., Helman, J. I., Wen, Y., Wang, C., and Li, L. (2011) CXCR4 promotes oral squamous cell carcinoma migration and invasion through inducing expression of MMP-9 and MMP-13 via the ERK signaling pathway. Mol. Cancer Res. 9, 161–172

52. Ingham, R. J., Gish, G., and Pawson, T. (2004) The Nedd4 family of E3 ubiquitin ligases. Functional diversity within a common modular architecture. Oncogene 23, 1972–1984

53. Jadwin, J. A., Rudd, V., Sette, P., Challa, S., and Bouamr, F. (2010) Late domain-independent rescue of a release-deficient Moloney murine leukemia virus by the ubiquitin ligase Itch. J. Virol. 84, 704–715

54. Chinni, S. R., Sivalogan, S., Dong, Z., Filho, J. C., Deng, X., Bonfil, R. D., and Cher, M. L. (2006) CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells. The role of bone microenvironment-associated CXCL12. Prostate 66, 32–48

55. Chinni, S. R., Yamamoto, H., Dong, Z., Sabbota, A., Bonfil, R. D., and Cher, M. L. (2008) CXCL12/CXCR4 transactivates HER2 in lipid rafts of prostate cancer cells and promotes growth of metastatic deposits in bone. Mol. Cancer Res. 6, 446–457

56. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004) Jun turnover is controlled through INK-dependent phosphorylation of the E3 ligase Itch. Science 306, 271–275

57. Ahn, Y. H., and Kurie, J. M. (2009) MKK4/SEK1 is negatively regulated through a feedback loop involving the E3 ubiquitin ligase Itch. J. Biol. Chem. 284, 29399–29404

58. Endo, K., Takeshita, T., Kasai, H., Sasaki, Y., Tanaka, N., Asao, H., Kikuchi, K., Yamada, M., Chenb, M., O’Shea, J. J., and Sugamura, K. (2000) STAM2, a new member of the STAM family, binding to the Janus kinases. FEBS Lett. 477, 55–61

59. Lohi, O., and Lehto, V. P. (1998) ITAM motif in an apoptosis receptor. Apoptosis 3, 335–336

60. Takeshita, T., Arita, T., Asao, H., Tanaka, N., Higuchi, M., Kuroda, H., Kaneko, K., Munakata, H., Endo, Y., Fujita, T., and Sugamura, K. (1996) Cloning of a novel signal-transducing adaptor molecule containing an SH3 domain and ITAM. Biochem. Biophys. Res. Commun. 225, 1035–1039

61. Takeshita, T., Arita, T., Higuchi, M., Asao, H., Endo, K., Kuroda, H., Tanaka, N., Murata, K., Ishii, N., and Sugamura, K. (1997) STAM, signal-transducing adaptor molecule, is associated with Janus kinases and involved in signaling for cell growth and c-myc induction. Immunity. 6, 449–457

62. Yamada, M., Ishii, N., Asao, H., Murata, K., Kanazawa, C., Sasah, S., and Sugamura, K. (2002) Signal-transducing adaptor molecules STAM1 and STAM2 are required for T-cell development and survival. Mol. Cell Biol. 22, 8648–8658

63. Rubin, J. B., Kung, A. L., Klein, R. S., Chan, J. A., Sun, Y., Schmidt, K., Kieran, M. W., Luster, A. D., and Segal, R. A. (2003) A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. Proc. Natl. Acad. Sci. U.S.A. 100, 13513–13518

64. Ren, J., Kee, Y., Huijbrgtsje, J. M., and Piper, R. C. (2007) Hse1, a component of the yeast Hrs-STAM ubiquitin-sorting complex, associates with ubiquitin peptides and a ligase to control sorting efficiency into multivesicular bodies. Mol. Biol. Cell 18, 324–335